



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 6 :</b> <b>C12N 9/24, 15/56</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/00528</b> <b>(43) International Publication Date:</b> 8 January 1998 (08.01.98)
<b>(21) International Application Number:</b> PCT/DK97/00283 <b>(22) International Filing Date:</b> 30 June 1997 (30.06.97) <b>(30) Priority Data:</b> 0715/96 28 June 1996 (28.06.96) DK 1004/96 17 September 1996 (17.09.96) DK <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S (DK/DK); Novo Allé, DK-2880 Bagsværd (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> WAHLEITHNER, Jill, Angela [US/US]; 1718 Tea Place, Davis, CA 95616 (US). FUGLSANG, Claus, Crone [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). HALKIER, Torben [DK/DK]; Novo Nordisk a/s, Novo Allé, DK- 2880 Bagsværd (DK). JOHANSEN, Charlotte [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). HANSEN, Mogens, Trier [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Novo Allé, Corporate Patents, DK-2880 Bagsværd (DK).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> A RECOMBINANT ENZYME WITH MUTANASE ACTIVITY		
<b>(57) Abstract</b> <p>The present invention relates to method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of a) isolating a DNA sequence encoding a mutanase from a filamentous fungus, b) introducing a kex2-site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, c) cloning the DNA sequence obtained in step b) into a suitable expression vector. The invention also relates to a recombinant expression vector comprising said mutanase gene sequence and a kex2 cleavage site between the DNA sequence encoding the pro-peptide and the region encoding the mature mutanase, a filamentous fungus host cell, a process for producing recombinant mutanase and a recombinant mutanase. It is also the object of the invention to provide compositions useful in oral care products for humans and animals.</p>		

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**Title:** A recombinant enzyme with mutanase activity

#### FIELD OF THE INVENTION

The present invention relates to a method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production, a recombinant expression vector comprising said mutanase gene sequence and a kex2 cleavage site between the DNA sequence encoding the pro-peptide and the DNA sequence encoding the mature mutanase, a filamentous fungus host cell, a process of producing recombinant mutanase, and said recombinant mutanase.

It is also the object of the invention to provide compositions useful in oral care products for humans and animals.

#### 15 BACKGROUND OF THE INVENTION

Mutanases are  $\alpha$ -1,3-glucanases (also known as  $\alpha$ -1,3-glucanohydrolases) which degrade the  $\alpha$ -1,3-glycosidic linkages in mutan. Mutanases have been described from two species of *Trichoderma* (Hasegawa et al., (1969), Journal of Biological Chemistry 20 244, p. 5460-5470; Guggenheim and Haller, (1972), Journal of Dental Research 51, p. 394-402) and from a strain of *Streptomyces* (Takehara et al., (1981), Journal of Bacteriology 145, p. 729-735), *Cladosporium resinae* (Hare et al. (1978), Carbohydrate Research 66, p. 245-264), *Pseudomonas* sp. (US patent no. 25 4,438,093), *Flavobacterium* sp. (JP 77038113), *Bacillus circulans* (JP 63301788) and *Aspergillus* sp.. A mutanase gene from *Trichoderma harzianum* has been cloned and sequenced (Japanese Patent No. 4-58889-A from Nissin Shokuhin Kaisha LTD).

Although mutanases have commercial potential for use as an antiplaque agent in dental applications and personal care products, e.g., toothpaste, chewing gum, or other oral and dental care products, the art has been unable to produce mutanases in significant quantities to be commercial useful.

US patent no. 4,353,891 (Guggenheim et al.) concerns plaque removal using mutanase produced by *Trichoderma harzianum* CBS 243.71 to degrade mutan synthesized by cultivating *Streptococcus*

*mutans* strain CBS 350.71 identifiable as OMZ 176.

It is an object of the present invention to provide a recombinant mutanase from *Trichoderma harzianum* which can be produced in commercially useful quantities.

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#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows plasmid pMT1796

Figure 2 shows plasmid construction of plasmids pMT1796, pMT1802, and pMT1815,

10 Figure 3 shows an outline of the construction of the *A. oryzae* recombinant mutanase expression vector pMT1802,

Figure 4 shows the pH-profile of recombinant and wild-type *T. harzianum* CBS 243.71 mutanase

Figure 5 shows the temperature profile of recombinant and wild-  
15 type *T. harzianum* CBS 243.71 mutanase at pH 7,

Figure 6 shows the temperature stability of recombinant and wild-type *T. harzianum* CBS 243.71 mutanase at pH 7,

Figure 7 shows the indirect Malthus standard curve for a mix  
20 culture of *S. mutans*, *A. viscosus* and *F. nucleatum* grown in BHI at 37°C.

#### SUMMARY OF THE INVENTION

The object of the invention is to provide a recombinant mutanase derived from a filamentous fungus by heterologous  
25 expression.

The present inventors have as the first been able to express the mutanase gene of a filamentous fungus heterologously and thus cleared the way for providing a single component, recombinant mutanase essentially free of any contaminants.

30 In the first aspect the invention relates to a method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of:

a) isolating a DNA sequence encoding a mutanase from a  
35 filamentous fungus,

b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the

mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 or kex2-like site of another fungal enzyme,

c) cloning the DNA sequence obtained in step b) into a suitable expression vector.

In a preferred embodiment the mutanase is obtained from a strain within the genus *Trichoderma*.

In step b) the mutanase (pre)pro-sequence may for instance be replaced with the Lipolase® (pre)pro-sequence or the TAKA- amylase (pre)pro-sequence.

It is also an object of the invention to provide an expression vector comprising a mutanase gene and a DNA sequence encoding a (pre)pro-peptide with a kex2 site or kex2-like site between the DNA sequences encoding said (pre)pro-peptide and the mature region of the mutanase.

The invention also relates to a filamentous host cell for production of recombinant mutanase derived from a filamentous fungus. Preferred host cells include filamentous fungi of the genera *Trichoderma*, *Aspergillus*, and *Fusarium*.

Further, the invention relates to a process for producing a recombinant mutanase in a host cell, comprising the steps:

a) transforming an expression vector comprising a mutanase gene with a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase into a suitable filamentous fungus host cell,

b) cultivating the host cell in a suitable culture medium under conditions permitting expression and secretion of an active mutanase,

c) recovering and optionally purifying the secreted active recombinant mutanase from the culture medium.

The expression vector may be prepared according to the above described method of the invention.

A recombinant mutanase may according to the invention be produced according to the process of the invention.

A substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any contaminants is also part of the invention.

The invention also relates to a composition comprising a recombinant mutanase of the invention or a substantially pure mutanase of the invention useful in oral care products and food, feed and/or pet food products.

5 Finally the invention relates to the use of the recombinant mutanase of the invention or the substantially purified mutanase of the invention or composition or product of the invention preventing the formation of human or animal dental plaque or removing dental plaque and for the use in food, feed and/or pet  
10 food products.

#### DETAILED DESCRIPTION OF THE INVENTION

The object of the invention is to provide a recombinant mutanase derived from a filamentous fungus by heterologous  
15 expression.

The present inventors have as the first been able to express the mutanase gene of a filamentous fungus heterologously and thus cleared the way for providing a single component recombinant mutanase essentially free of any contaminants.

20 The principle of the invention can be used for all mutanases derivable from filamentous fungi, such as from filamentous fungi of the genus *Trichoderma*, such a strain of *Trichoderma harzianum*, especially *Trichoderma harzianum* CBS 243.71, and the genera *Streptomyces*, *Cladosporium* or *Aspergillus*.

25 Previously it has not been possible to produce mutanases of filamentous fungi heterologously. Consequently, according to prior art mutanases are produced homologously and comprise a mixture of other enzyme activities besides the mutanase (i.e. with undesired contaminants).

30 An example of this is *Trichoderma harzianum* CBS 243.71 which are known to produce a mutanase as also described above. The mutanase derived from *Trichoderma harzianum* CBS 243.71 has before the successful findings of the present invention only been produced homologously.

35 It is advantageous to be able to produce the mutanase heterologously, as it is then possible to provide a single component mutanase free of undesired contaminants. Further, it

facilitates providing an isolated and purified enzyme of the invention in industrial scale.

According to the invention it is possible to express mutanases derived from filamentous fungi in a suitable host cell by introducing a kex2 cleavage site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 site or kex2-like site of another fungal enzyme.

The (pre)pro-sequence have for instance be the Lipolase® (pre)pro-sequence or the TAKA-amylase (pre)pro-sequence.

### Pro-peptides

A large number of mature proteins are initially synthesised with a N-terminal extension, the pro-peptide, varying from very small peptides (e.g. GLA 6 amino acids) to relatively long peptides (e.g. PEPA 49 amino acids).

The pro-peptide can perform a number of different functions. Firstly, pro-peptides might contribute to the efficiency of co-translational translocation of the protein across the ER-membrane. Secondly, pro-peptides might contribute to co-translational proteolytic processing of the polypeptide. Thirdly, they might act as intracellular targeting signal for routing to specific cellular compartments. Fourthly, in some pro-proteins the pro-peptide keeps the protein inactive until it reaches its site of action.

Removal of the pro-peptide from the mature protein occurs in general by processing by a specific endopeptidase, usually after the two positively charged amino acid residues Arg-Arg, Arg-Lys or Lys-Arg. However, also other amino acid combinations, containing at least one basic amino acid, have been found to be processed.

The absence of these doublets in mature, endogenous secreted proteins might protect them from proteolytic cleavage. As di-basic cleavage is thought to occur in the Golgi, the internal di-basic peptide sequences in cytoplasmic proteins will not be attacked by this processing.

### Kex2 sites

Kex2 sites (see e.g. Methods in Enzymology Vol 185, ed. D. Goeddel, Academic Press Inc. (1990), San Diego, CA, "Gene Expression Technology") and kex2-like sites are di-basic recognition sites (i.e. cleavage sites) found between the pro-peptide encoding region and the mature region of some proteins.

Insertion of a kex2 site or a kex2-like site have in certain cases been shown to improve correct endopeptidase processing at the pro-peptide cleavage site resulting in increased protein secretion levels.

However, in a number of other cases insertion of a Kex2 cleavage site did not increase the secretion level. For instance, Cullen et al., (1987), Bio/Technology, vol. 5, p. 369-376, found that insertion of a kex2 site in the secretion signal of chymosin (i.e. signal peptide and pro-peptide), which encoded the glucoamylase signal peptide and pro-peptide fused to prochymosin, did not increase the secretion level of recombinant chymosin expressed in a *Aspergillus nidulans* host cell.

Other examples of references showing that insertion of a kex2 site or a kex2-like site do not always increase the secretion level include Valverde et al., (1995), J. of Biolog. Chem, p. 15821-15826)

In the context of the present invention the term "heterologous" production means expression of a recombinant enzyme in an host organism different from the original donor organism or expression of a recombinant enzyme by the donor organism.

The term "homologous" production means expression of the wild-type enzyme by the original organism.

In the first aspect the invention relates to a method for construction of an expression vector comprising a mutanase gene suitable for heterologous production comprising the steps of:

a) isolating a DNA sequence encoding a mutanase from a filamentous fungus known to produce a mutanase,

b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 or kex2-like site of  
5 another fungal enzyme,

c) cloning the mutanase gene with the kex2 site or kex2-like site obtained in step b) into a suitable expression vector.

In a preferred embodiment of the mutanase gene is obtained from the genus *Trichoderma*, preferably a strain of the species  
10 *T. harzianum*, especially the strain *T. harzianum* CBS 243.71.

The complete mutanase gene DNA sequence derived from *Trichoderma harzianum* CBS 243.71 is shown in SEQ ID No. 1

In step b) the mutanase (pre)pro-sequence may for instance be replaced with the Lipolase® (pre)pro-sequence or the TAKA-  
15 amylase (pre)pro-sequence.

In the examples below illustrating the present invention a kex2-site is inserted into the *Trichoderma harzianum* mutanase gene presented in SEQ ID No. 1 as the site specific mutation  
E36 → K36.

20

#### Isolation of the mutanase gene

The DNA sequence encoding a mutanase may, in accordance with well-known procedures, conveniently be isolated from DNA from a suitable source, such as any of the above mentioned organisms  
25 known to comprise a mutanase gene, by use of synthetic oligonucleotide probes prepared on the basis of the DNA sequence disclosed herein.

For instance, a suitable oligonucleotide probe may be prepared on the basis of the nucleotide sequences shown in SEQ ID  
30 no. 1 or the amino acid sequence shown in SEQ ID no. 2 or any suitable sub-sequence thereof.

According to this method primers are designed from the knowledge to at least a part of SEQ ID No. 2. Fragments of mutanase gene are then PCR amplified by the use of these  
35 primers. These fragments are used as probes for cloning the complete gene.

Alternatively, the DNA sequence encoding a mutanase may be isolated by a general method involving

- cloning, in suitable vectors, a DNA or cDNA library from a strain of genus *Trichoderma*,
- 5 - transforming suitable host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the DNA library,
- screening for positive clones by determining any mutanase activity of the enzyme produced by such clones, and
- 10 - isolating the DNA coding an enzyme from such clones.

The general method is further disclosed in WO 93/11249 the contents of which are hereby incorporated by reference.

#### 15 **Expression vector**

In another aspect the invention relates to an expression vector comprising a mutanase gene and a DNA sequence encoding a pro-peptide with a kex2 site or kex2-like site inserted between the DNA sequences encoding said pro-peptide and the mature region of the mutanase.

In preferred embodiments of the invention the expression vector comprises besides the kex2 site or kex2-like site an operably linked DNA sequence encoding a prepro-peptide (i.e. signal peptide and a pro-peptide). The prepro-sequence may advantageously be the original mutanase signal-sequence or the Lipolase® signal-sequence or the TAKA signal-sequence and the original mutanase pro-sequence or the Lipolase® pro-sequence or the TAKA pro-sequence.

The promoter may be the TAKA promoter or the TAKA:TPI promoter.

In a specific embodiment of the invention the expression vector is the pMT1796 used to illustrate the concept of the invention in Example 3 below.

The choice of vector will often depend on the host cell into which it is to be introduced.

Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the

replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the mutanase should also be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

The procedures used to ligate the DNA sequences coding for the mutanase, a prepro-sequence including the kex2 site or kex2-like site, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

## 20 Host Cell

A third aspect of the invention relates to a filamentous fungi host cell for production of recombinant mutanase derived from a filamentous fungus of the genus *Trichoderma*, such as a strain of *T. harzianum*, especially *T. harzianum* CBS 243.71, or the genus *Aspergillus*, such as a strain of *A. oryzae* or *A. niger*, or a strain of the genus *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gibberella zeae*, previously *Sphaeria zeae*, synonym with *Gibberella roseum* and *Gibberella roseum* f. sp. *cerealis*), or *Fusarium sulphureum* (in the perfect state named *Gibberella puricaris*, synonym with *Fusarium trichothecioides*, *Fusarium bactridioides*, *Fusarium sambucium*, *Fusarium roseum*, and *Fusarium roseum* var. *graminearum*), *Fusarium cerealis* (synonym with *Fusarium crockwellense*) or *Fusarium venenatum*.

The host cell may advantageously be a *F. graminearum* described in WO 96/00787 (from Novo Nordisk A/S), e.g. the strain deposited as *Fusarium graminearum* ATCC 20334. The strain ATCC

20334 was previously wrongly classified as *Fusarium graminearum* (Yoder, W. and Christianson, L. 1997). RAPD-based and classical taxonomic analyses have now revealed that the true identity of the Quorn fungus, ATCC 20334, is *Fusarium venenatum* Nirenburg  
5 sp. nov.

In a preferred embodiment of the invention the host cell is a protease deficient or protease minus strain.

This may for instance be the protease deficient strain *Aspergillus oryzae* JaL125 having the alkaline protease gene  
10 named "alp" deleted. This strain is described in PCT/DK97/00135 (from Novo Nordisk A/S).

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a  
15 manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

According to a further aspect the invention relates to a process for producing a recombinant mutanase in a host cell. Said  
20 process comprises the following steps:

- a) transforming an expression vector encoding a mutanase gene with a kex2 site or a kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase into a suitable filamentous fungus host cell,
- 25 b) cultivating the host cell in a suitable culture medium under conditions permitting the expression of the expression vector,
- c) recovering the secreted recombinant mutanase from the culture medium,
- d) and optionally purifying the recombinant mutanase.

30 The recombinant expression vector may advantageously be any of the above described.

Further, the filamentous fungi host cells to be used for production of the recombinant mutanase of the invention according to the process of the invention may be any of the  
35 above mentioned host cell, especially of the genera *Aspergillus*, *Fusarium* or *Trichoderma*.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed mutanase is secreted into the culture medium and may be recovered from there by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

It is also an important object of the invention to provide a recombinant mutanase produced according to the process of the invention.

The isolated recombinant mutanase has essentially an amino acid sequence as shown in SEQ ID no. 2. From SDS-PAGE a molecular weight around 80 kDa was found.

The pH optimum of the recombinant mutanase was found to lie in the range from 3.5 to 5.5 which equals the pH optimum of the wild-type mutanase (see Figure 4). The temperature optimum of both the recombinant and wild-type mutanase was found to be around 45°C at pH 7 and around 55°C at pH 5.5 (see Figure 5). Further, the residual activity starts to decline at 40°C at pH 7, while the enzyme is more stable at pH 5.5, where the residual activity starts to decline at 55°C.

The inventors have also provided a substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any active contaminants, such as other enzyme activities.

#### Composition

It is also an object of the invention to provide a composition comprising the recombinant mutanase of the invention or the purified wild-type mutanase essentially free of any active contaminants of the invention.

35

#### Oral care composition

In a still further aspect, the present invention relates to an oral care composition useful as an ingredient in oral care products.

An oral care composition of the invention may suitably  
5 comprise an amount of the recombinant *Trichoderma harzianum*  
mutanase equivalent to an enzyme activity, calculated as enzyme  
activity units in the final oral care product, in the range from  
0.001 MU to 1000 MU/ml, preferably from 0.01 MU/ml to 500 MU/ml,  
such as from 0.1 MU/ml to 100 MU/ml, especially 0.05 MU/ml to 100  
10 MU/ml.

It is also contemplated according to the invention to include other enzyme activities than mutanase activity in the oral care composition. Contemplated enzyme activities include activities from the group of enzymes comprising dextranases,  
15 oxidases, such as glucose oxidase, L-amino acid oxidase, peroxidases, such as e.g. the *Coprinus sp.* peroxidases described in WO 95/10602 (from Novo Nordisk A/S) or lactoperoxidase or, haloperoxidases, laccases, proteases, such as papain, acidic  
protease (e.g. the acidic proteases described in WO 95/02044  
20 (Novo Nordisk A/S)), endoglucosidases, lipases, amylases, including amyloglucosidases, such as AMG (from Novo Nordisk A/S), and mixtures thereof.

#### Oral care products

25 The oral care product may have any suitable physical form (i.e. powder, paste, gel, liquid, ointment, tablet etc.). An "oral care product" can be defined as a product which can be used for maintaining or improving the oral hygiene in the mouth of humans and animals, by preventing dental caries, preventing the  
30 formation of dental plaque and tartar, removing dental plaque and tartar, preventing and/or treating dental diseases etc.

At least in the context of the present invention oral care products do also encompass products for cleaning dentures, artificial teeth and the like.

35 Examples of such oral care products include toothpaste, dental cream, gel or tooth powder, odontic, mouth washes, pre- or post brushing rinse formulations, chewing gum, lozenges, and candy.

Toothpastes and tooth gels typically include abrasive polishing materials, foaming agents, flavouring agents, humectants, binders, thickeners, sweetening agents, whitening/bleaching/ stain removing agents, water, and optionally 5 enzymes.

Mouth washes, including plaque removing liquids, typically comprise a water/alcohol solution, flavour, humectant, sweetener, foaming agent, colorant, and optionally enzymes.

#### 10 Abrasives

Abrasive polishing material might also be incorporated into the dentifrice product of the invention. According to the invention said abrasive polishing material includes alumina and hydrates thereof, such as alpha alumina trihydrate, magnesium 15 trisilicate, magnesium carbonate, kaolin, aluminosilicates, such as calcined aluminum silicate and aluminum silicate, calcium carbonate, zirconium silicate, and also powdered plastics, such as polyvinyl chloride, polyamides, polymethyl methacrylate, polystyrene, phenol-formaldehyde resins, melamine-formaldehyde 20 resins, urea-formaldehyde resins, epoxy resins, powdered polyethylene, silica xerogels, hydrogels and aerogels and the like. Also suitable as abrasive agents are calcium pyrophosphate, water-insoluble alkali metaphosphates, dicalcium phosphate and/or its dihydrate, dicalcium orthophosphate, 25 tricalcium phosphate, particulate hydroxyapatite and the like. It is also possible to employ mixtures of these substances.

Dependent on the oral care product the abrasive product may be present in from 0 to 70% by weight, preferably from 1% to 70%. For toothpastes the abrasive material content typically lies in 30 the range of from 10% to 70% by weight of the final toothpaste product.

Humectants are employed to prevent loss of water from e.g. toothpastes. Suitable humectants for use in oral care products according to the invention include the following compounds and 35 mixtures thereof: glycerol, polyol, sorbitol, polyethylene glycols (PEG), propylene glycol, 1,3-propanediol, 1,4-butanediol, hydrogenated partially hydrolysed polysaccharides and the like.

Humectants are in general present in from 0% to 80%, preferably 5 to 70% by weight in toothpaste.

Silica, starch, tragacanth gum, xanthan gum, extracts of Irish moss, alginates, pectin, cellulose derivatives, such as hydroxyethyl cellulose, sodium carboxymethyl cellulose and hydroxypropyl cellulose, polyacrylic acid and its salts, polyvinylpyrrolidone, can be mentioned as examples of suitable thickeners and binders, which helps stabilizing the dentifrice product. Thickeners may be present in toothpaste creams and gels in an amount of from 0.1 to 20% by weight, and binders to the extent of from 0.01 to 10% by weight of the final product.

#### Foaming agents

As foaming agent soap, an-ionic, cat-ionic, non-ionic, amphoteric and/or zwitterionic surfactants can be used. These may be present at levels of from 0% to 15%, preferably from 0.1 to 13%, more preferably from 0.25 to 10% by weight of the final product.

#### Surfactants

Surfactants are only suitable to the extent that they do not exert an inactivation effect on the present enzymes. Surfactants include fatty alcohol sulphates, salts of sulphonated mono-glycerides or fatty acids having 10 to 20 carbon atoms, fatty acid-albumen condensation products, salts of fatty acids amides and taurines and/or salts of fatty acid esters of isethionic acid.

#### Sweetening agents

Suitable sweeteners include saccharin.

#### Flavouring agents

Flavours, such as spearmint, are usually present in low amounts, such as from 0.01% to about 5% by weight, especially from 0.1% to 5%.

### Whitening/bleaching agents

Whitening/bleaching agents include  $H_2O_2$  and may be added in amounts less than 5%, preferably from 0.25 to 4%, calculated on the basis of the weight of the final product.

- 5 The whitening/bleaching agents may be an enzyme, such as an oxidoreductase. Examples of suitable teeth bleaching enzymes are described in WO 97/06775 (from Novo Nordisk A/S).

### Water

- 10 Water is usually added in an amount giving e.g. toothpaste a flowable form.

### Additional agents

- Further water-soluble anti-bacterial agents, such as  
15 chlorhexidine digluconate, hexetidine, alexidine, quaternary ammonium anti-bacterial compounds and water-soluble sources of certain metal ions such as zinc, copper, silver and stannous (e.g. zinc, copper and stannous chloride, and silver nitrate), may also be included.
- 20 Also contemplated according to the invention is the addition of compounds which can be used as fluoride source, dyes/colorants, preservatives, vitamins, pH-adjusting agents, anti-carries agents, desensitizing agents etc.

### 25 Enzymes

- Other essential components used in oral care products and in oral care products of the invention are enzymes. Enzymes are biological catalysts of chemical reactions in living systems. Enzymes combine with the substrates on which they act forming an  
30 intermediate enzyme-substrate complex. This complex is then converted to a reaction product and a liberated enzyme which continue its specific enzymatic function.

- Enzymes provide several benefits when used for cleansing of the oral cavity. Proteases break down salivary proteins, which  
35 are adsorbed onto the tooth surface and form the pellicle, the first layer of resulting plaque. Proteases along with lipases destroy bacteria by lysing proteins and lipids which form the

structural components of bacterial cell walls and membranes. Dextranase breaks down the organic skeletal structure produced by bacteria that forms a matrix for bacterial adhesion. Proteases and amylases, not only prevents plaque formation, but also prevents the development of calculus by breaking-up the carbohydrate-protein complex that binds calcium, preventing mineralization.

#### Toothpaste

- 10 A toothpaste produced from an oral care composition of the invention (in weight % of the final toothpaste composition) may typically comprise the following ingredients:

Abrasive material	10 to 70%
Humectant	0 to 80%
15 Thickener	0.1 to 20%
Binder	0.01 to 10%
Sweetener	0.1% to 5%
Foaming agent	0 to 15%
Whitener	0 to 5%
20 Enzymes	0.0001% to 20%

In a specific embodiment of the invention the oral care product is toothpaste having a pH in the range from 6.0 to about 8.0 comprising

- |                   |                   |
|-------------------|-------------------|
| a) 10% to 70%     | Abrasive material |
| 25 b) 0 to 80%    | Humectant         |
| c) 0.1 to 20%     | Thickener         |
| d) 0.01 to 10%    | Binder            |
| e) 0.1% to 5%     | Sweetener         |
| f) 0 to 15%       | Foaming agent     |
| 30 g) 0 to 5%     | Whitener          |
| i) 0.0001% to 20% | Enzymes.          |

Said enzymes referred to under i) include the recombinant mutanase of the invention, and optionally other types of enzymes mentioned above known to be used in toothpastes and the like.

### Mouth wash

A mouth wash produced from an oral care composition of the invention (in weight % of the final mouth wash composition) may typically comprise the following ingredients:

- 0-20% Humectant
- 0-2% Surfactant
- 0-5% Enzymes
- 0-20% Ethanol
- 10 0-2% Other ingredients (e.g. flavour, sweetener  
active ingredients such as fluorides).
- 0-70% Water

The mouth wash composition may be buffered with an appropriate buffer e.g. sodium citrate or phosphate in the pH-range 6-7.5.

- 15 The mouth wash may be in none-diluted form (i.e. must be diluted before use).

### Method of Manufacture

The oral care composition and products of the present invention can be made using methods which are common in the oral product area.

According to the present invention the recombinant mutanase and/or the substantially purified mutanase free of active contaminants can be use in food, feed and/or pet food products.

25

## **MATERIALS AND METHODS**

### Materials

#### Micro-organisms

- 30 *Trichoderma harzianum* CBS 243.71

- A. *oryzae* JaL 125: *Aspergillus oryzae* IFO 4177 available from Institute for Fermentation, Osaka; 17-25 Juso Hammachi 2-Chome Yodogawa-ku, Osaka, Japan, having the alkaline protease gene named "alp" (described by Murakami K et al., (1991), Agric. Biol. Chem. 55, p. 2807-2811) deleted by a one step gene replacement method (described by G. May in "Applied Molecular Genetics of Filamentous Fungi" (1992), p. 1-25. Eds. J. R. Kinghorn and G.

Turner; Blackie Academic and Professional), using the *A. oryzae* pyrG gene as marker.

*E. coli* DH5 $\alpha$

#### 5 Plasmids and Vectors:

pMT1796 (Figure 1 and Figure 2)

pMT1802 (Figure 2)

pMT1815 (Figure 2)

pHD414: *Aspergillus* expression vector is a derivative of the  
10 plasmid p775 (described in EP 238.023). The construction of the  
pHD414 is further described in WO 93/11249. pHD414 contains the  
*A. niger* glucoamylase terminator and the *A. oryzae* TAKA amylase  
promoter.

pHD414+mut (Figure 3)

15 pHan37 containing the TAKA:TPI promoter

#### Linkers:

Linker #1:

20 GATCCTCACATG TTG GGC GTT GTC CGC CGT CTA GGC CTA GG  
GAGTGT TAC AAC CCG CAA CAG GCT GCA GAT CCG GAT CCG C  
Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly

Linker #2:

25 C CAA TAC TGT TAG T  
GT ACG GTT ATG ACA ATC AGATC  
Ala Cys Gln Tyr Cys \*\*\*

#### Primers:

Primer 1: 5' GGGGGGATCCACCATGAG 3' (SEQ ID No. 3)

30 Primer 2: 5' ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC 3' (SEQ ID  
No. 4)

Primer 3: 5' GCCAGTCCTATTCGTCGAGCTTCTTCTGCTGACCGT 3' (SEQ ID  
No. 5)

Primer 4: 5' CCACGGTCACCAACAATAC 3' (SEQ ID No. 6)

35 Primer 5: GGGGGGATCCACCATGAG (SEQ ID No. 7),

Primer 6: ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC (SEQ ID No. 8)

Primer 7: GCCAGTCCTATTCGTCGAGCTTCTTCTGCTGACCGT (SEQ ID NO. 9),

Primer 8: CCACGGTCACCAACAATAC (SEQ ID No. 10).

Enzymes:

lysyl-specific protease from *Achromobacter*

*Trichoderma harzianum* CBS 243.71 fermentation broth (Batch no.  
5 PPM 3897)

Media, Substrates and Solutions:

YPM: 2% maltose, 1% bactopectone and 0.5% yeast extract)

DAPI: 4',6-diamidino-2-phenylindole (Sigma D-9542)

10 Britton-Robinson Buffer

BHI: Brain Heart Infusion broth

Equipment:

10 kDa cut-off ultra-filtration cassette (Alpha Minisette from  
15 Filtron).

Phenyl-sepharose FF (high sub) column (Pharmacia)

Seitz EK1 filter plate

Q-sepharose FF column (Pharmacia)

Applied Biosystems 473A protein sequencer

20 2 litre Kieler fermenter

Olympus model BX50 microscope

Malthus Flexi M2060 (Malthus Instrument Limited)

Methods:25 **Molecular biology procedures**

All molecular biology procedures including restriction digests, DNA ligations, *E. coli* transformations, DNA isolations, Southern hybridizations, PCR amplifications, and library constructions and screenings were completed using standard techniques (Sambrook, J., Fritsch, E. F., and Maniatis, T.  
30 1989. *Molecular cloning: A laboratory manual*/E.F. Cold Spring Harbor Laboratory Press, Plainview, NY).

**Preparation of Mutan**

35 Mutan is prepared by growing *Streptococcus mutans* CBS 350.71 at pH 6.5, 37°C (kept constant), and with an aeration rate of 75 rpm in a medium comprised of the following components:

	NZ-Case	6.5 g/litre
	Yeast Extract	6 g/litre
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20 g/litre
	K <sub>2</sub> PO <sub>4</sub>	3 g/litre
5	Glucose	50 g/litre
	Pluronic PE6100	0.1%

After 35 hours, sucrose is added to a final concentration of 60 g/litre to induce glucosyltransferase. The total fermentation time is 75 hours. The supernatant from the fermentation is centrifuged and filtered (sterile). Sucrose is then added to the supernatant to a final concentration of 5% (pH is adjusted to pH 7.0 with acetic acid) and the solution is stirred overnight at 37°C. The solution is filtered and the insoluble mutan is harvested on propex and washed extensively with deionized water containing 1% sodium benzoate, pH 5 (adjusted with acetic acid). Finally, the insoluble mutan is lyophilized and ground.

#### Determination of mutanase activity (MU)

One Mutanase Unit (MU) is the amount of enzyme which under standard conditions liberates 1  $\mu$ mol reducing sugar (calculated as glucose) per minute. Reducing sugars were measured with alkaline K<sub>3</sub>Fe(CN)<sub>6</sub>.

#### Standard Conditions

Substrate.....1.5% mutan  
25 Reaction time.....15 minutes  
Temperature.....40°C  
pH.....5.5

A detailed description of Novo Nordisk's analytical method (AF 180/1-GB) is available from Novo Nordisk A/S on request.

#### Mutanase Plate Assay

A 5% mutan suspension is made in 50 mM sodium acetate, pH 5.5 and the suspension is homogenised for 15 minutes in an Ultra Turrax T25 homogenizer at 4°C. 1% agarose in 50 mM sodium acetate, pH 5.5 is made 0.2% with respect to mutan and 12.5 ml agarose is casted in each petri dish (d=10 cm). The sample to be

analyzed for mutanase activity is applied in sample wells punched in the agarose, and the plate is incubated overnight at 37°C, whereafter clearing zones are formed around mutanase containing samples.

5

#### Western hybridization

Western hybridizations are performed using the ECL western blotting system (Amersham International, plc, Buckinghamshire, England) and a primary antibody solution containing polyclonal rabbit-anti-mutanase. The limit of detection is 0.001 MU/ml.

#### Mass spectrometry

Mass spectrometry of purified wild-type mutanase is done using matrix assisted laser desorption ionization time-of-flight mass spectrometry in a VG Analytical ToFSpec. For mass spectrometry 2 ml of sample is mixed with 2 ml saturated matrix solution (a-cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile (70:30)) and 2 ml of the mixture spotted onto the target plate. Before introduction into the mass spectrometer the solvent is removed by evaporation. Samples are desorbed and ionized by 4 ns laser pulses (337 nm) at threshold laser power and accelerated into the field-free flight tube by an accelerating voltage of 25 kV. Ions are detected by a microchannel plate set at 1850 V.

#### 25 Preparation of Hydroxyapatite disks (HA)

Hydroxyapatite tablets are prepared by compressing 250 mg of hydroxyapatite in a tablet die at about 5,900 kg (13,000 lbs) of pressure for 5 minutes. The tablets are then sintered at 600°C for 4 hours and finally hydrated with sterile deionized water.

30

#### Plaque coating of Hydroxyapatite disks (HA)

Hydroxyapatite disks (HA) were dry sterilised (121°C, 2 bar, 20 minutes) and coated with filter sterilised saliva for 18 hours at 37°C. The HA disks were placed in a sterile rack in a beaker, Brain Heart Infusion broth (BHI) containing 0.2% sucrose was poured into the beaker covering the disks. Sterile Na<sub>2</sub>S (pH 7.0) was added immediately before inoculation given the final concen-

tration of 5 g/litre. A mixture 1:1:1 of *Streptococcus mutans*, *Actinomyces viscosus* and *Fusobacterium nucleatum* grown anaerobically (BHI, 37°C, 24 h) was used as inoculum in the concentration of approximately  $10^6$  cfu/ml. The disks were incubated 5 anaerobic at 37°C for 4 days with slight stirring.

#### Malthus-method for plaque

The Malthus-method is based on the methods described in Johnston et al., (1995), Journal of Microbiological Methods 21, 10 p. 15-26 and Johansen et al. (1995), Journal of Applied Bacteriology 78, p. 297-303.

#### EXAMPLES

##### 15 Example 1

##### Purification of wild-type Mutanase

100 g fermentation broth of *Trichoderma harzianum* CBS 243.71 (Batch no. PPM 3897) were dissolved in 1 litre 10 mM sodium acetate, pH 5.2 overnight at 4°C.

20 65 g DEAE-Sephadex A-50 were swelled in 3 litre 10 mM sodium acetate, pH 5.2. Excess buffer was removed after swelling. DEAE-Sephadex was mixed with the crude mutanase preparation for 1 hour and unbound material was collected by filtration through Propex cloth. The gel was further washed with 2.5 l of 10 mM sodium acetate, pH 5.2. A pool containing the unbound material was made; 25 volume 4 litre. Remaining DEAE-Sephadex particles were removed by filtration through a Whatman GF/F filter.

350 ml S-Sepharose was equilibrated in 10 mM sodium acetate, pH 5.2 and mixed with 600 ml of the pool from the DEAE-Sephadex 30 for 10 minutes. Unbound material was collected by filtration through Propex cloth and the gel was washed with 500 ml 10 mM sodium acetate buffer, pH 5.2. Bound material was eluted with the same buffer containing 1 M NaCl. The procedure was repeated 7 times. The combined pool containing the unbound material (7 35 litre) was concentrated on a Filtron concentrator equipped with a 10 kDa cut-off membrane and followed by a buffer change to 10 mM sodium acetate, pH 4.7. The concentrate was filtrated through a

Whatmann GF/F filter. The final volume of the concentrate was 600 ml.

An S-Sepharose column (180 ml, 2.6 x 33 cm) was equilibrated with 10 mM sodium acetate, pH 4.7. The pH adjusted concentrate  
5 from the S-Sepharose batch ion exchange was applied onto the column in 50 ml portions with a flow of 10 ml/min. The mutanase was eluted with a linear gradient from 0 to 20 mM NaCl in 3 column volumes. The residual protein was eluted with the same buffer containing 1 M NaCl. Fractions were analyzed for mutanase  
10 activity (plate assay) and fractions with high activity were pooled. The procedure was repeated 12 times. The combined mutanase pool was concentrated in a Filtron concentrator equipped with a 10 kDa cut-off membrane and followed by a buffer change to 10 mM Tris-HCl, pH 8.0. The final volume of the concentrate was  
15 870 ml.

The concentrated pool from the S-Sepharose column was further purified on a HiLoad Q-Sepharose column (50 ml, 2.6 x 10 cm) equilibrated with 10 mM Tris-HCl, pH 8.0. Portions of 130 ml was applied with a flow of 8 ml/min. Elution of the mutanase was per-  
20 formed with a linear gradient from 0 to 50 mM NaCl in 12 column volumes. Fractions with high mutanase activity (plate assay) were pooled, concentrated in an Amicon cell equipped with a 10 kDa cut-off membrane. Finally, the mutanase preparation was dialyzed extensively against 10 mM sodium phosphate, pH 7.0 and filtrated  
25 through a 0.45 mm filter.

The yield of the mutanase in the purification described above was 300 mg. The purity of the HiLoad-Q preparation was analyzed by SDS-PAGE and N-terminal sequencing and judged by both methods the purity was around 95%.

## Example 2

### N-terminal sequencing of wild-type Mutanase

N-terminal amino acid sequencing was carried out in an Applied Biosystems 473A protein sequencer.

35 To generate peptides reduced and S-carboxymethylated mutanase (> 450 mg) was digested with the lysyl-specific protease from *Achromobacter* (10 mg) in 20 mM  $\text{NH}_4\text{HCO}_3$  for 16 hours at 37°C. The

resulting peptides were separated by reversed phase HPLC using a Vydac C<sub>18</sub> column eluted with a linear gradient of 80% 2-propanol containing 0.08% TFA in 0.1% aqueous TFA. Peptides were repurified by reversed-phase-HPLC using a Vydac C<sub>18</sub> column eluted with linear gradients of 80% acetonitrile containing 0.08% TFA in 0.1% aqueous TFA before being subjected to N-terminal amino acid sequencing.

The amino acid sequences determined are given below.

N-terminal:

10 Ala-Ser-Ser-Ala-Asp-Arg-Leu-Val-Phe-Cys-His-Phe-Met-Ile-Gly-Ile-Val-Gly-Asp-Arg-Gly-Ser-Ser-Ala-Asp-Tyr-Asp-Asp-Asp-  
Peptide 1:

Val Phe-Ile-Ser-Phe-Asp-Phe-Asn-Trp-Trp-Ser-Pro-Gly-Asn-Ala-Val-Gly-Val-Gly-Gln-Lys

15 Peptide 2:

Pro-Tyr-Leu-Ala-Pro-Val-Ser-Pro-Trp-Phe-Phe-Thr-His-Phe-Gly-Pro-Glu-Val-Ser-Tyr-Ser-

Peptide 3:

Trp-Val-Asn-Asp-Met-Pro-His-Asp-Gly-Phe-Leu-Asp-Leu-Ser-Lys

20

### Example 3

**Construction of the mutanase expression vectors, pMT1796, pMT1802 and pMT1815**

A cDNA clone encoding mutanase was identified in a  
25 *Trichoderma harzianum* CBS 243.71 library by hybridization with a fragment of the gene amplified by PCR using primers based on the mutanase sequence shown in SEQ ID NO. 1.

DNA sequence analysis of the isolated clone, pHD414+mut, showed that it indeed encoded the mutanase gene, and that the  
30 5' end of the construct contained a long leader sequence. To remove this leader, pHD414+mut was restricted with the enzymes *EcoRI*, *NarI* and *XhoI*. From this digestion a 3499 nt (nucleotide) vector fragment and a 610 nt *NarI/XhoI* fragment were isolated. These two fragments were then ligated with  
35 linker #1 (see above) and a 618 nt *EcoRI/BamHI* fragment from pHan37 containing the TAKA:TPI promoter, giving plasmid pJW99. HD414+mut was next digested with *XhoI* and *SphI*, and a 1790 nt

fragment encoding amino acids 35-598 of the mutanase gene was isolated.

This fragment was ligated with linker #2 (see above) and pJW99 that had been linearized with the restriction enzymes *Xba*I and *Xho*I. The resulting plasmid, pMT1802, contains the *T. harzianum* mutanase gene under the control of the TAKA:TPI promoter. Plasmid pMT1796 is identical to pMT1802 except that E36 of the mutanase protein has been changed to K36 by replacing the *Xho*I/*Kpn*I fragment of pMT1802 with a PCR amplified fragment containing the desired mutation.

This PCR fragment was created in a two step procedure as reported in Ho, et al. (1989), Gene, 77, p. 51-59, using the following primers:

- Primer 1 (nt 2751 5' CAGCGTCCACATCAGAGC nt 2769) and  
15 Primer 2 (nt 3306 5' GAAGAAGCACGTTTCTCGAGAGACCG nt 3281);  
Primer 3 (nt 3281 5' CGGTCTCTGAGAAACGTGCTTCTTC nt 3306) and  
Primer 4 (nt 4266 5' GCCACTTCCGTTATTAGCC nt 4248); nucleotide numbers refer to the pMT1802 plasmid (See SEQ ID No. 11).

To create pMT1815, a 127 nt DNA fragment was PCR amplified using again a two step procedure and the primers:

- Primer 5: GGGGGGATCCACCATGAG;  
Primer 6: ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC;  
Primer 7: GCCAGTCCTATTCGTCGAGCTTCTTCTGCTGACCGT;  
Primer 8: CCACGGTCACCAACAATAC,  
25 and the plasmids pHan37 and pMT1802 as templates in the first round of amplification.

This fragment contains a *Bam*HI restriction enzyme site followed by the Lipolase® prepro-sequence in frame with residues 38-54 of the mutanase protein and ending with a *Bst*EII site.  
30

The fragment was digested with the restriction enzymes *Bst*EII and *Bam*HI and inserted into pMT1802 that had been linearized with the same pair of enzymes. Changes in constructs were confirmed and the integrity of the resulting coding regions were checked by nucleotide sequencing.  
35

**Example 4****Expression of recombinant Mutanase in *Aspergillus oryzae***

The strain *A. oryzae* JaL125 was transformed using a PEG-mediated protocol (see EP 238 023) and a DNA mixture containing 0.5 µg of a plasmid encoding the gene that confers resistance to the herbicide Basta and 8.0 µg of one of the three mutanase expression plasmids. Transformants were selected on minimal plates containing 0.5% basta and 50 mM urea as a nitrogen source.

**Shake flask cultures**

Transformed colonies were spore purified twice on selection media and spores were harvested. A 20 ml universal container (Nunc, cat #364211) containing 10 ml YPM (2% maltose, 1% bactopectone and 0.5% yeast extract) was inoculated with spores and grown for 5 days with shaking at 30°C. The supernatant was harvested after 5 days growth.

Construct	highest mutanase level detected	number of transformants tested
pMT1802, mutanase prepro + mutanase	<0.001	10
pMT1796, mutanase prepro + KEX2 + mutanase	3.8	4
pMT1815, Lipolase® prepro + mutanase	0.16	22

Table 1 Comparison of mutanase expression from the three different expression constructs. The limit of detection was 0.001 MU/ml

The presence of mutanase in culture supernatants was examined by western hybridizations. SDS-PAGE and protein transfers were performed using standard protocols.

**Example 5****Purification of recombinant mutanase**

700 ml fermentation broth was filtered and concentrated. The pH was adjusted to 4.7 (conductivity around 300 µS/cm) and the broth was loaded onto an S-Sepharose column (XK 50/22) (Pharmacia) equilibrated in 10 mM sodium acetate pH 4.7. The

mutanase was eluted in a linear NaCl gradient. The major part of the mutanase appeared in the unbound fractions. These fractions were pooled and concentrated. Then the concentrate was loaded onto a HiLoad Q-Sepharose column (Pharmacia) equilibrated in 10 mM Tris-HCl, pH 8.0 (around 600  $\mu$ S/cm). The mutanase was eluted in a linear gradient of NaCl and the mutanase containing-fractions were pooled according to purity and activity. The pooled fractions were concentrated and a fraction was further purified by gel filtration on a Superdex 75 (16/60) column (Pharmacia) in sodium acetate pH 6.0.

The purified mutanase has a specific activity around 19 MU pr. absorption unit at 280 nm. From SDS-PAGE (Novex 4-20 %; run according to the manufacturer's instructions) a molecular weight around 80 kDa is found.

The N-terminal amino acid sequence was confirmed to be identical to the N-terminal amino acid sequence of the wt mutanase (Ala-Ser-Ser-Ala-) (see Example 2)

#### Example 6

##### pH-profile of mutanase

500 ml 5 % mutan in 50 mM Britton-Robinson buffer at varying pH was added 2 ml enzyme sample (diluted in MilliQ-filtered water) in large vials (to ensure sufficient agitation) and incubated for 15 minutes at 40°C while shaking vigorously. The reaction was terminated by adding 0.5 ml 0.4 M NaOH and the samples were filtered on Munktell filters. 100  $\mu$ l filtrate in Eppendorf vials were added 750  $\mu$ l ferricyanide reagent (0.4 g/l  $K_3Fe(CN)_6$ , 20 g/l  $Na_2CO_3$ ) and incubated 15 minutes at 85°C. After allowing the samples to cool, the decrease in absorption at 420 nm was measured. A dilution series of glucose was included as a standard. Substrate and enzyme blanks were always included. Samples were run in duplicate. The pH-optimum for both wild-type and recombinant enzyme is around pH 3.5-5.5 (see Figure 4).

#### Example 7

**Temperature profile of mutanase:**

500 ml 5 % mutan in 100 mM sodium acetate, pH 5.5 or in 100 mM sodium phosphate, pH 7 was added. 2 ml enzyme sample (diluted in MilliQ-filtered water) in large vials (to ensure sufficient agitation) and incubated for 15 minutes at various temperatures while shaking vigorously. The reaction was terminated by adding 0.5 ml 0.4 M NaOH and the samples were filtered on Munktell filters. 100 µl filtrate in Eppendorf vials were added 750 µl ferricyanide reagent (0.4 g/l  $K_3Fe(CN)_6$ , 20 g/l  $Na_2CO_3$ ) and incubated 15 minutes at 85°C. After allowing the samples to cool, the drop in absorption at 420 nm was measured. A dilution series of glucose was included as a standard. Substrate and enzyme blanks were always included. Samples were run in duplicate. The temperature profiles for the recombinant and wt mutanase were identical. The temperature optimum at pH 7 was around 45 °C. The temperature optimum at pH 5.5 was above 55° (See Figure 5).

**Example 8****20 Temperature stability of mutanase:**

The temperature stability was investigated by pre-incubating enzyme samples for 30 minutes at various temperatures in 0.1 M sodium acetate, pH 5.5 or in 0.1 M sodium phosphate, pH 7 before assaying the residual activity. Both recombinant and wt mutanase have similar temperature stability profiles. The residual activity starts to decline at 40 °C at pH 7, while the enzyme is more stable at pH 5.5, where the residual activity starts to decline at 55°C (See Figure 6).

**30 Example 9****Molecular weight of purified wild-type Mutanase**

The mass spectrometry, performed as described above, of the mutanase revealed an average mass around 75 kDa. In addition, it was clear from the spectra that the glycosylation of the mutanase is heterogeneous. The peptide mass of the mutanase is more than 64 kDa meaning that more than 11 kDa of carbohydrate is attached

to the enzyme.

#### Example 10

##### Activity of mutanase against Dental Plaque

5 A plaque biofilm was grown anaerobic on saliva coated hydroxyapatite disks as described in the Material and Methods Section above. The plaque was a mixed culture of *Streptococcus mutans* (SFAG; CBS 350.71), *Actinomyces viscosus* (DSM 43329) and *Fusobacterium nucleatum* subsp. *polymorphum* (DSM 20482).

10 HA disks with plaque were transferred to acetate buffer (pH 5.5) containing recombinant *Trichoderma* mutanase 1 MU/ml and whirled for 2 minutes (sterile buffer was used as control).

After enzyme treatment, the disks were either DAPI stained or transferred to Malthus cells, as indirect impedance measurements  
15 were used when enumerating living adherent cells (Malthus Flexi M2060, Malthus Instrument Limited).

For the impedance measurements 3 ml of BHI were transferred to the outer chamber of the indirect Malthus cells, and 0.5 ml of sterile KOH (0.1 M) was transferred to the inner chamber. After  
20 mutanase treatment the disks with plaque were slightly rinsed with phosphate buffer and transferred to the outer chamber. The detection times (dt) in Malthus were converted to colony counts by use of a calibration curve relating cfu/ml to dt (Figure 7).

The calibration curve was constructed by a series of 10-fold  
25 dilution rate prepared from the mixed culture. Conductance dt of each dilution step was determined in BHI and a calibration curve relating cfu/ml of the 10 fold dilutions to dt in BHI was constructed for the mixed culture (Figure 7).

The removal of plaque from the disks was also determined by  
30 fluorescent microscopy, after mutanase treatment disks were stained with DAPI (3 mM) and incubated in the dark for 5 minutes (20°C). The DAPI stained cells were examined with the x.100 oil immersion fluorescence objective on an Olympus model BX50 microscope equipped with a 200 W mercury lamp and an UV- filter.

35 The result was compared with the quantitative data obtained by the impedance measurements.

The number of living cells on the saliva treated HA-surface after enzyme treatment was determined by the Malthus method and shown in Table 1. However, by the Malthus method it is not possible to distinguish between a bactericidal activity of mutanase or an enzymatic removal of the plaque. Therefore a decrease in living bacteria on the surface has to be compared with the simultaneously removal of plaque from the surface which is estimated by the DAPI staining.

Mutanase (MU/ml)	Log <sub>10</sub> reduction (cfu/cm <sup>2</sup> )	Removal of plaque (%)	No. of observations
0	0	0	10
1	1.4	96	6

10 Table 2: Enzymatic plaque removal (pH 5.5, 2 minutes) from saliva treated hydroxyapatite determined by impedance measurements.

A significant removal of plaque was determined by fluorescent microscopy after treatment with mutanase. Thus mutanase reduced the amount of adhering cells. However, the activity was observed as a removal of plaque and not as a bactericidal activity against cells in plaque.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Novo Nordisk A/S  
 (B) STREET: Novo Alle  
 (C) CITY: Bagsvaerd  
 (E) COUNTRY: Denmark  
 (F) POSTAL CODE (ZIP): DK-2880  
 (G) TELEPHONE: +45 4444 8888  
 (H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: A recombinant enzyme with mutanase activity

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1905 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(B) STRAIN: Trichoderma harzianum CBS 243.71

(ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 1..1905  
 (A) NAME/KEY: sig peptide  
 (B) LOCATION: 1..120

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG	TTG	GGC	GTT	GTC	CGC	CGT	CTA	GGC	CTA	GGC	GCC	CTT	GCT	GCC	GCA	48
Met	Leu	Gly	Val	Val	Arg	Arg	Leu	Gly	Leu	Gly	Ala	Leu	Ala	Ala	Ala	
1				5				10					15			
GCT	CTG	TCT	TCT	CTC	GGC	AGT	GCC	GCT	CCC	GCC	AAT	GTT	GCT	ATT	CGG	96
Ala	Leu	Ser	Ser	Leu	Gly	Ser	Ala	Ala	Pro	Ala	Asn	Val	Ala	Ile	Arg	
			20				25					30				
TCT	CTC	GAG	GAA	CGT	GCT	TCT	TCT	GCT	GAC	CGT	CTC	GTA	TTC	TGT	CAC	144
Ser	Leu	Glu	Glu	Arg	Ala	Ser	Ser	Ala	Asp	Arg	Leu	Val	Phe	Cys	His	
		35				40					45					
TTC	ATG	ATT	GGT	ATT	GTT	GGT	GAC	CGT	GGC	AGC	TCA	GCA	GAC	TAT	GAT	192
Phe	Met	Ile	Gly	Ile	Val	Gly	Asp	Arg	Gly	Ser	Ser	Ala	Asp	Tyr	Asp	
	50					55				60						
GAT	GAC	ATG	CAA	CGT	GCC	AAA	GCC	GCT	GGC	ATT	GAC	GCA	TTC	GCT	CTG	240
Asp	Asp	Met	Gln	Arg	Ala	Lys	Ala	Ala	Gly	Ile	Asp	Ala	Phe	Ala	Leu	
	65			70					75					80		
AAC	ATC	GGC	GTT	GAC	GGC	TAT	ACC	GAC	CAG	CAA	CTC	GGG	TAT	GCC	TAT	288
Asn	Ile	Gly	Val	Asp	Gly	Tyr	Thr	Asp	Gln	Gln	Leu	Gly	Tyr	Ala	Tyr	
			85					90					95			
GAC	TCT	GCC	GAC	CGT	AAT	GGC	ATG	AAA	GTC	TTC	ATT	TCA	TTC	GAT	TTC	336
Asp	Ser	Ala	Asp	Arg	Asn	Gly	Met	Lys	Val	Phe	Ile	Ser	Phe	Asp	Phe	
		100				105						110				
AAC	TGG	TGG	AGC	CCC	GGT	AAT	GCA	GTT	GGT	GTT	GGC	CAG	AAG	ATT	GCG	384
Asn	Trp	Trp	Ser	Pro	Gly	Asn	Ala	Val	Gly	Val	Gly	Gln	Lys	Ile	Ala	
	115					120					125					

CAG TAT GCC AGC CGT CCC GCC CAG CTG TAT GTT GAC AAC CGG CCA TTC Gln Tyr Ala Ser Arg Pro Ala Gln Leu Tyr Val Asp Asn Arg Pro Phe 130 135 140	432
GCC TCT TCC TTC GCT GGT GAC GGT TTG GAT GTA AAT GCG TTG CGC TCT Ala Ser Ser Phe Ala Gly Asp Gly Leu Asp Val Asn Ala Leu Arg Ser 145 150 155 160	480
GCT GCA GGC TCC AAC GTT TAC TTT GTG CCC AAC TTC CAC CCT GGT CAA Ala Ala Gly Ser Asn Val Tyr Phe Val Pro Asn Phe His Pro Gly Gln 165 170 175	528
TCT TCC CCC TCC AAC ATT GAT GGC GCC CTC AAC TGG ATG GCC TGG GAT Ser Ser Pro Ser Asn Ile Asp Gly Ala Leu Asn Trp Met Ala Trp Asp 180 185 190	576
AAT GAT GGA AAC AAC AAG GCA CCC AAG CCG GGC CAG ACT GTC ACG GTG Asn Asp Gly Asn Asn Lys Ala Pro Lys Pro Gly Gln Thr Val Thr Val 195 200 205	624
GCA GAC GGT GAC AAC GCT TAC AAG AAT TGG TTG GGT GGC AAG CCT TAC Ala Asp Gly Asp Asn Ala Tyr Trp Leu Gly Lys Pro Tyr 210 215 220	672
CTA GCG CCT GTC TCC CCT TGG TTT TTC ACC CAT TTT GGC CCT GAA GTT Leu Ala Pro Val Ser Pro Trp Phe Phe Thr His Phe Gly Pro Glu Val 225 230 235 240	720
TCA TAT TCC AAG AAC TGG GTC TTC CCA GGT GGT CCT CTG ATC TAT AAC Ser Tyr Ser Lys Asn Trp Val Phe Pro Gly Gly Pro Leu Ile Tyr Asn 245 250 255	768
CGG TGG CAA CAG GTC TTG CAG CAG GGC TTC CCC ATG GTT GAG ATT GTT Arg Trp Gln Val Leu Gln Gln Gly Phe Pro Met Val Glu Ile Val 260 265 270	816
ACC TGG AAT GAC TAC GGC GAG TCT CAC TAC GTC GGT CCT CTG AAG TCT Thr Trp Asn Asp Tyr Gly Glu Ser His Tyr Val Gly Pro Leu Lys Ser 275 280 285	864
AAG CAT TTC GAT GAT GGC AAC TCC AAA TGG GTC AAT GAT ATG CCC CAT Lys His Phe Asp Asp Gly Asn Ser Lys Trp Val Asn Asp Met Pro His 290 295 300	912
GAT GGA TTC TTG GAT CTT TCA AAG CCG TTT ATT GCT GCA TAT AAG AAC Asp Gly Phe Leu Asp Leu Ser Lys Pro Phe Ile Ala Ala Tyr Lys Asn 305 310 315 320	960
AGG GAT ACT GAT ATA TCT AAG TAT GTT CAA AAT GAG CAG CTT GTT TAC Arg Asp Thr Asp Ile Ser Lys Tyr Val Gln Asn Glu Gln Leu Val Tyr 325 330 335	1008
TGG TAC CGC CGC AAC TTG AAG GCA TTG GAC TGC GAC GCC ACC GAC ACC Trp Tyr Arg Arg Asn Leu Lys Ala Leu Asp Cys Asp Ala Thr Asp Thr 340 345 350	1056
ACC TCT AAC CGC CCG GCT AAT AAC GGA AGT GGC AAT TAC TTT ATG GGA Thr Ser Asn Arg Pro Ala Asn Asn Gly Ser Gly Asn Tyr Phe Met Gly 355 360 365	1104
CGC CCT GAT GGT TGG CAA ACT ATG GAT GAT ACC GTT TAT GTT GCC GCA Arg Pro Asp Gly Trp Gln Thr Met Asp Asp Thr Val Tyr Val Ala Ala 370 375 380	1152
CTT CTC AAG ACC GCC GGT AGC GTC ACG GTC ACG TCT GGC GGC ACC ACT Leu Leu Lys Thr Ala Gly Ser Val Thr Val Thr Ser Gly Gly Thr Thr 1200	1200

385	390	395	400	
CAA ACG TTC CAG GCC AAC GCC GGA GCC AAC CTC TTC CAA ATC CCT GCC Gln Thr Phe Gln Ala Asn Ala Gly Ala Asn Leu Phe Gln Ile Pro Ala	405	410	415	1248
AGC ATC GGC CAG CAA AAG TTT GCT CTA ACT CGC AAC GGT CAG ACC GTC Ser Ile Gly Gln Lys Phe Ala Leu Thr Arg Asn Gly Gln Thr Val	420	425	430	1296
TTT AGC GGA ACC TCA TTG ATG GAT ATC ACC AAC GTT TGC TCT TGC GGT Phe Ser Gly Thr Ser Leu Met Asp Ile Thr Asn Val Cys Ser Cys Gly	435	440	445	1344
ATC TAC AAT TTC AAC CCA TAT GTT GGC ACC ATT CCT GCC GGC TTT GAC Ile Tyr Asn Phe Asn Pro Tyr Val Gly Thr Ile Pro Ala Gly Phe Asp	450	455	460	1392
GAC CCT CTT CAG GCT GAC GGT CTT TTC TCT TTG ACC ATC GGA TTG CAT Asp Pro Leu Gln Ala Asp Gly Leu Phe Ser Leu Thr Ile Gly Leu His	465	470	475	1440
GTC ACG ACT TGT CAG GCC AAG CCA TCT CTT GGA ACC AAC CCT CCT GTC Val Thr Thr Cys Gln Ala Lys Pro Ser Leu Gly Thr Asn Pro Pro Val	485	490	495	1488
ACT TCT GGC CCT GTG TCC TCG CTG CCA GCT TCC TCC ACC ACC CGC GCA Thr Ser Gly Pro Val Ser Ser Leu Pro Ala Ser Ser Thr Thr Arg Ala	500	505	510	1536
TCC TCG CCT CCT GTT TCT TCA ACT CGT GTC TCT TCT CCC CCT GTC TCT Ser Ser Pro Pro Val Ser Ser Thr Arg Val Ser Ser Pro Pro Val Ser	515	520	525	1584
TCC CCT CCA GTT TCT CGC ACC TCT TCT CCC CCT CCC CCT CCG GCC AGC Ser Pro Pro Val Ser Arg Thr Ser Ser Pro Pro Pro Pro Ala Ser	530	535	540	1632
AGC ACG CCG CCA TCG GGT CAG GTT TGC GTT GCC GGC ACC GTT GCT GAC Ser Thr Pro Pro Ser Gly Gln Val Cys Val Ala Gly Thr Val Ala Asp	545	550	555	1680
GGC GAG TCC GGC AAC TAC ATC GGC CTG TGC CAA TTC AGC TGC AAC TAC Gly Glu Ser Gly Asn Tyr Ile Gly Leu Cys Gln Phe Ser Cys Asn Tyr	565	570	575	1728
GGT TAC TGT CCA CCG GGA CCG TGT AAG TGC ACC GCC TTT GGT GCT CCC Gly Tyr Cys Pro Pro Gly Pro Cys Lys Cys Thr Ala Phe Gly Ala Pro	580	585	590	1776
ATC TCG CCA CCG GCA AGC AAT GGG CGC AAC GGC TGC CCT CTA CCG GGA Ile Ser Pro Pro Ala Ser Asn Gly Arg Asn Gly Cys Pro Leu Pro Gly	595	600	605	1824
GAA GGC GAT GGT TAT CTG GGC CTG TGC AGT TTC AGT TGT AAC CAT AAT Glu Gly Asp Gly Tyr Leu Gly Leu Cys Ser Phe Ser Cys Asn His Asn	610	615	620	1872
TAC TGC CCG CCA ACG GCA TGC CAA TAC TGT TAG Tyr Cys Pro Pro Thr Ala Cys Gln Tyr Cys *	625	630	635	1905

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 635 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly Ala Leu Ala Ala Ala
 1           5           10          15
Ala Leu Ser Ser Leu Gly Ser Ala Ala Pro Ala Asn Val Ala Ile Arg
          20          25          30
Ser Leu Glu Glu Arg Ala Ser Ser Ala Asp Arg Leu Val Phe Cys His
          35          40          45
Phe Met Ile Gly Ile Val Gly Asp Arg Gly Ser Ser Ala Asp Tyr Asp
          50          55          60
Asp Asp Met Gln Arg Ala Lys Ala Ala Gly Ile Asp Ala Phe Ala Leu
          65          70          75          80
Asn Ile Gly Val Asp Gly Tyr Thr Asp Gln Gln Leu Gly Tyr Ala Tyr
          85          90          95
Asp Ser Ala Asp Arg Asn Gly Met Lys Val Phe Ile Ser Phe Asp Phe
          100         105         110
Asn Trp Trp Ser Pro Gly Asn Ala Val Gly Val Gly Gln Lys Ile Ala
          115         120         125
Gln Tyr Ala Ser Arg Pro Ala Gln Leu Tyr Val Asp Asn Arg Pro Phe
          130         135         140
Ala Ser Ser Phe Ala Gly Asp Gly Leu Asp Val Asn Ala Leu Arg Ser
          145         150         155         160
Ala Ala Gly Ser Asn Val Tyr Phe Val Pro Asn Phe His Pro Gly Gln
          165         170         175
Ser Ser Pro Ser Asn Ile Asp Gly Ala Leu Asn Trp Met Ala Trp Asp
          180         185         190
Asn Asp Gly Asn Asn Lys Ala Pro Lys Pro Gly Gln Thr Val Thr Val
          195         200         205
Ala Asp Gly Asp Asn Ala Tyr Lys Asn Trp Leu Gly Gly Lys Pro Tyr
          210         215         220
Leu Ala Pro Val Ser Pro Trp Phe Phe Thr His Phe Gly Pro Glu Val
          225         230         235         240
Ser Tyr Ser Lys Asn Trp Val Phe Pro Gly Gly Pro Leu Ile Tyr Asn
          245         250         255
Arg Trp Gln Gln Val Leu Gln Gln Gly Phe Pro Met Val Glu Ile Val
          260         265         270
Thr Trp Asn Asp Tyr Gly Glu Ser His Tyr Val Gly Pro Leu Lys Ser
          275         280         285
Lys His Phe Asp Asp Gly Asn Ser Lys Trp Val Asn Asp Met Pro His
          290         295         300
Asp Gly Phe Leu Asp Leu Ser Lys Pro Phe Ile Ala Ala Tyr Lys Asn
          305         310         315         320
Arg Asp Thr Asp Ile Ser Lys Tyr Val Gln Asn Glu Gln Leu Val Tyr
          325         330         335
Trp Tyr Arg Arg Asn Leu Lys Ala Leu Asp Cys Asp Ala Thr Asp Thr

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340	345	350
Thr Ser Asn Arg Pro Ala Asn Asn Gly Ser Gly Asn Tyr Phe Met Gly		
355	360	365
Arg Pro Asp Gly Trp Gln Thr Met Asp Asp Thr Val Tyr Val Ala Ala		
370	375	380
Leu Leu Lys Thr Ala Gly Ser Val Thr Val Thr Ser Gly Gly Thr Thr		
385	390	395
Gln Thr Phe Gln Ala Asn Ala Gly Ala Asn Leu Phe Gln Ile Pro Ala		
405	410	415
Ser Ile Gly Gln Gln Lys Phe Ala Leu Thr Arg Asn Gly Gln Thr Val		
420	425	430
Phe Ser Gly Thr Ser Leu Met Asp Ile Thr Asn Val Cys Ser Cys Gly		
435	440	445
Ile Tyr Asn Phe Asn Pro Tyr Val Gly Thr Ile Pro Ala Gly Phe Asp		
450	455	460
Asp Pro Leu Gln Ala Asp Gly Leu Phe Ser Leu Thr Ile Gly Leu His		
465	470	475
Val Thr Thr Cys Gln Ala Lys Pro Ser Leu Gly Thr Asn Pro Pro Val		
485	490	495
Thr Ser Gly Pro Val Ser Ser Leu Pro Ala Ser Ser Thr Thr Arg Ala		
500	505	510
Ser Ser Pro Pro Val Ser Ser Thr Arg Val Ser Ser Pro Pro Val Ser		
515	520	525
Ser Pro Pro Val Ser Arg Thr Ser Ser Pro Pro Pro Pro Ala Ser		
530	535	540
Ser Thr Pro Pro Ser Gly Gln Val Cys Val Ala Gly Thr Val Ala Asp		
545	550	555
Gly Glu Ser Gly Asn Tyr Ile Gly Leu Cys Gln Phe Ser Cys Asn Tyr		
565	570	575
Gly Tyr Cys Pro Pro Gly Pro Cys Lys Cys Thr Ala Phe Gly Ala Pro		
580	585	590
Ile Ser Pro Pro Ala Ser Asn Gly Arg Asn Gly Cys Pro Leu Pro Gly		
595	600	605
Glu Gly Asp Gly Tyr Leu Gly Leu Cys Ser Phe Ser Cys Asn His Asn		
610	615	620
Tyr Cys Pro Pro Thr Ala Cys Gln Tyr Cys *		
625	630	635

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Primer 1"

CAGCGTCCAC ATCACGAGC

19

- (2) INFORMATION FOR SEQ ID NO: 4:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 26 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer 2"

GAAGAAGCAC GTTCTGCAG AGACCG

26

- (2) INFORMATION FOR SEQ ID NO: 5:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 26 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer 3"

CGGTCTCTCG AGAAACGTGC TTCTTC

26

- (2) INFORMATION FOR SEQ ID NO: 6:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 19 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer 4"

GCCACTTCCG TTATTAGCC

19

- (2) INFORMATION FOR SEQ ID NO: 7:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 18 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer 5"

GGGGGGATCC ACCATGAG

18

- (2) INFORMATION FOR SEQ ID NO: 8:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 36 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer 6"

ACGGTCAGCA GAAGAAGCTC GACGAATAGG ACTGGC

36

- (2) INFORMATION FOR SEQ ID NO: 9:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 36 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single

- (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "Primer 7"

GCCAGTCCTA TTCGTCGAGC TTCTTCTGCT GACCGT

36

- (2) INFORMATION FOR SEQ ID NO: 10:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "Primer 8"

CCACGGTCAC CAACAATAC

19

- (2) INFORMATION FOR SEQ ID NO: 11:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 6032 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (vi) ORIGINAL SOURCE:  
 (B) STRAIN: *Trichoderma harzianum* CBS 243.71  
 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 3188..5092  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GACGAAAGGG	CCTCGTGATA	CGCCTATTTT	TATAGGTTAA	TGTCATGATA	ATAATGGTTT	60
CTTAGACGTC	AGGTGGCACT	TTTCGGGGAA	ATGTGCGCGG	AACCCCTATT	TGTTTATTTT	120
TCTAAATACA	TTCAAATATG	TATCCGCTCA	TGAGACAATA	ACCCTGATAA	ATGCTTCAAT	180
AATATTGAAA	AAGGAAGAGT	ATGAGTATTC	AACATTTCCG	TGTCGCCCTT	ATTCCTTTT	240
TTGCGGCATT	TTGCCTTCCT	GTTTTTGCTC	ACCCAGAAAC	GCTGGTGAAA	GTAAGAGATG	300
CTGAAGATCA	GTTGGGTGCA	CGAGTGGGTT	ACATCGAACT	GGATCTCAAC	AGCGGTAAGA	360
TCCTTGAGAG	TTTTCGCCCC	GAAGAACGTT	TTCCAATGAT	GAGCACTTTT	AAAGTTCTGC	420
TATGTGGCGC	GGTATTATCC	CGTATTGACG	CCGGGCAAGA	GCAACTCGGT	CGCCGCATAC	480
ACTATTCTCA	GAATGACTTG	GTTGAGTACT	CACCACTCAC	AGAAAAGCAT	CTTACGGATG	540
GCATGACAGT	AAGAGAATTA	TGCAGTGCTG	CCATAACCAT	GAGTGATAAC	ACTGCGGCCA	600
ACTTACTTCT	GACAAACGATC	GGAGGACCGA	AGGAGCTAAC	CGCTTTTTTG	CACAACATGG	660
GGGATCATGT	AATTCGCCCT	GATCGTTGGG	AACCGGAGCT	GAATGAAGCC	ATACCAAGAC	720
ACGAGCGTGA	CACCAACGATG	CCTGTAGCAA	TGGCAACAAC	GTTGCGCAAA	CTATTAACTG	780
GCGAACTACT	TACTCTAGCT	TCCCGGCAAC	AATTAATAGA	CTGGATGGAG	GCGGATAAAG	840
TTGCAGGACC	ACTTCTGCGC	TCGGCCCTTC	CGGCTGGCTG	GTTTATTGCT	GATAAATCTG	900
GAGCCGGTGA	GCCTGGGTCT	CGCGGTATCA	TTGCAGCACT	GGGGCCAGAT	GGTAAGCCCT	960
CCGTGTACGT	AGCTTACTAC	ACGACGGGGA	GTCAAGCAAC	TATGGATGAA	CGAAATAGAC	1020
AGATCGCTGA	GATAGGTGCC	TCACTGATTA	AGCATTGGTA	ACTGTCAGAC	CAAGTTTACT	1080
CATATATACT	TAGATTGAT	TTAAACTTCT	ATTTTAAAT	TAAAAGGATC	TAGGTGAAGA	1140
TCCTTTTTGA	TAATCTCATG	ACCAAAATCC	CTTAACGTGA	GTTTTTCGTC	CACCTAGCGT	1200
CAGACCCCGT	AGAAAAGATC	AAAGGATCTT	CTTGAGATCC	TTTTTTCTG	CGCGTAATCT	1260
GCTGCTTGCA	AACAAAAAAA	CCACCCTCTAC	CAGCGGTGGT	TGTTTTCGCG	GATCAAGAGC	1320
TACCAACTCT	TTTTCCGAAG	GTAACCTGGCT	TCAGCAGAGC	GCAGATACCA	AATACTGTCC	1380
TTCTAGTGTA	CCGCTAGTTA	GGCCACCATT	TCAAGAACTC	TGTAGCAGCC	CCTACATACC	1440
TCGCTCTGCT	AATCCTGTGA	CCAGTGGCTG	CTGCCAGTGG	CGATAAGTCG	TGCTTATCCG	1500
GGTTGGACTC	AAGACGATAG	TTACC GGATA	AGGCGCAGCG	GTCGGGCTGA	ACGGGGGGTT	1560
CGTGACACAC	CGCCAGCTTG	GAGCCGAACG	CCTACACCGA	ACTGAGATAC	CTACAGCGTG	1620
AGCATTGAGA	AAGCGCCACG	CTTCCCGAAG	GGAGAAAGGC	GGACAGGTAT	CCGTAAGCG	1680

GCAGGGTCGG	AACAGGAGAG	CGCACGAGGG	AGCTTCCAGG	GGGAAACGCC	TGGTATCTTT	1740
ATAGTCCTGT	CGGGTTTCGC	CACCTCTGAC	TTGAGCGTCG	ATTTTTGTGA	TGCTCGTCAG	1800
GGGGGCGGAG	CCTAGGAAA	AACGCCAGCA	ACGCGGCCCT	TTTACGGTTC	CTGGCCCTTT	1860
GCTGGCCTTT	TGCTCACATG	TTCTTTCCTG	CGTTATCCCC	TGATTCTGTG	GATAACCGTA	1920
TTACCCGCTT	TGAGTGAGCT	GATACCGCTC	GCCGCAGCCG	AACGACCGAG	CGCAGCGAGT	1980
CAGTGAGCGA	GGAAAGCGAA	GAGCGCCCAA	TACGCAAACC	GCCTCTCCCC	GCGCGTGGCG	2040
CGATTTCATTA	ATGCAGCCTG	ATTAATGATT	ACATACGCCT	CCGGGTAGTA	GACCGAGCAG	2100
CCGAGCGAGT	TCAGCGCCTA	AAACGCCTTA	TACAATTAAG	CAGTTAAAGA	AGTTAGAATC	2160
TACGCTTAAA	AAGCTACTTA	AAAATCGATC	TCGCACTCCC	GATTTCGCTA	TCAAAACCGA	2220
TTTAAATCAA	CTGATTAAAG	GTGCCGAACG	AGCTATAAAT	GATATAACAA	TATTAAGCA	2280
TTAATTAGAG	CAATATCAGG	CCGCGCACGA	AAGGCAACTT	AAAAAGCGAA	AGCGCTCTAC	2340
TAAACAGATT	ACTTTTAAAA	AAGGCACATC	AGTATTTAAA	GCCCGAATCC	TTATTAAGCG	2400
CCGAAATCAG	GCAGATAAAG	CCATACAGGC	AGATAGACCT	CTACCTATTA	AATCGGCTTC	2460
TAGGCGCGCT	CCATCTAAAT	GTTCTGGCTG	TGGTGTACAG	GGGCATAAAA	TTACGCACTA	2520
CCCGAATCGA	TAGAACTACT	CATTTTATA	TAGAAGTCAG	CACTCATAGT	GTTTTGATCA	2580
TTTAAATTT	TTATATGGCG	GGTGGTGGCG	AACTCGTTTG	CGCGGGCAAC	TCGCTTACCG	2640
ATTACGTTAG	GGCTGATATT	TACGTAAAA	TCGTCAAGGG	ATGCAAGACC	AAAGTAGTAA	2700
AAACCCGGAA	GTCACAGACA	TCCAAGCCCA	AGTCCTTCAC	GGAGAAACCC	CAGCGTCCAC	2760
ATCACGAGCG	AAGGACCAACC	TCTAGGCATC	GGACGCACCA	TCCAATTGCA	AGCAGCAAAG	2820
CGAAACAGCC	CAAGAAAAAG	GTCCGCCCGT	CGGCCCTTTC	TGCAACGCTG	ATCACGGGCA	2880
GGCATCCAAC	CAACACCCCTC	CAGAGTGACT	AGGGGCGGAA	ATTTAAAGGG	ATTAATTTC	2940
ACTCAACACC	AAATACACAGT	CGTCCCCGGT	ATTGTCTCTG	AGAAATGCAAT	TTAAACTCTT	3000
CTGCCAATCG	CTTGGATTCC	CCCGCCCTAG	TCGTAGAGCT	TAAAGTATGT	CCCTTGTCGA	3060
TGCGATGTAT	CACAACATAT	AAATACTAGC	AAGGGATGCC	ATGCTTGGAG	TTTCCAATC	3120
AATTTACCTC	TATCCCAACT	TCTCTTCCTT	CCTCAATCCT	CTATATACAC	AACTGGGGAT	3180
CCTCACA ATG	TTG GGC GTT	GTC CGC CGT	CTA GGC	CTA GGC	GCC CTT	3229
Met	Leu	Gly	Val	Val	Arg	Arg
1	5	10	15	20	25	30
GCC GCA GCT	CTG TCT TCT	CTC GGC AGT	GCC GCT CCC	GCC AAT GTT	GCT	3277
Ala Ala Ala	Leu Ser Ser	Leu Gly Ser	Ala Ala Pro	Ala Asn Val	Ala	
15	20	25	30	35	40	45
ATT CGG TCT	CTC GAG GAA	CGT GCT TCT	TCT GCT GAC	CGT CTC GTA	TTC	3325
Ile Arg Ser	Leu Glu Glu	Arg Ala Ser	Ser Ala Asp	Arg Leu Val	Phe	
50	55	60	65	70	75	80
TGT CAC TTC	ATG ATT GGT	ATT GTT GGT	GAC CGT GGC	AGC TCA GCA	GAC	3373
Cys His Phe	Met Ile Gly	Ile Val Gly	Asp Arg Gly	Ser Ser Ala	Asp	
80	85	90	95	100	105	110
TAT GAT GAT	GAC ATG CAA	CGT GCC AAA	GCC GCT GGC	ATT GAC GCA	TTC	3421
Tyr Asp Asp	Asp Met Gln	Arg Ala Lys	Ala Ala Gly	Ile Asp Ala	Phe	
115	120	125	130	135	140	145
GCT CTG AAC	ATC GGC GTT	GAC GGC TAT	ACC GAC CAG	CAA CTC GGG	TAT	3469
Ala Leu Asn	Ile Gly Val	Asp Gly Tyr	Thr Asp Gln	Gln Leu Gly	Tyr	
150	155	160	165	170	175	180
GCC TAT GAC	TCT GCC GAC	CGT AAT GGC	ATG AAA GTC	TTC ATT TCA	TTC	3517
Ala Tyr Asp	Ser Ala Asp	Arg Asn Gly	Met Lys Val	Phe Ile Ser	Phe	
185	190	195	200	205	210	215
GAT TTC AAC	TGG TGG AGC	CCC GGT AAT	GCA GTT GGT	GTT GGC CAG	AAG	3565
Asp Phe Asn	Trp Trp Ser	Pro Gly Asn	Ala Val Gly	Val Gly Gln	Lys	
220	225	230	235	240	245	250
ATT GCG CAG	TAT GCC AGC	CGT CCC GCC	CAG CTG TAT	GTT GAC AAC	CGG	3613
Ile Ala Gln	Tyr Ala Ser	Arg Pro Ala	Gln Leu Tyr	Val Asp Asn	Arg	
255	260	265	270	275	280	285
CCA TTC GCC	TCT TCC TTC	GCT GGT GAC	GGT TTG GAT	GTA AAT GCG	TTG	3661
Pro Phe Ala	Ser Ser Phe	Ala Gly Asp	Gly Leu Asp	Val Asn Ala	Leu	
290	295	300	305	310	315	320
CGC TCT GCT	GCA GGC TCC	AAC GTT TAC	TTT GTG CCC	AAC TTC CAC	CCT	3709
Arg Ser Ala	Ala Gly Ser	Asn Val Tyr	Phe Val Pro	Asn Phe His	Pro	
325	330	335	340	345	350	355
GGT CAA TCT	TCC CCC TCC	AAC ATT GAT	GGC GCC CTC	AAC TGG ATG	GCC	3757
Gly Gln Ser	Ser Ser Pro	Ser Asn Ile	Asp Gly Ala	Leu Asn Trp	Met	
360	365	370	375	380	385	390
TGG GAT AAT	GAT GGA AAC	AAC AAG GCA	CCC AAG CCG	GGC CAG ACT	GTC	3805
Trp Asp Asn	Asp Gly Asn	Asn Lys Ala	Pro Lys Pro	Gly Gln Thr	Val	
395	400	405	410	415	420	425
ACG GTG GCA	GAC GGT GAC	AAC GCT TAC	AAG AAT TGG	TTG GGT GGC	AAG	3853
Thr Val Ala	Asp Gly Asp	Asn Ala Tyr	Lys Asn Trp	Leu Gly Gly	Lys	
210	215	220	225	230	235	240

CCT	TAC	CTA	GCG	CCT	GTC	TCC	CCT	TGG	TTT	TTC	ACC	CAT	TTT	GGC	CCT	3901
Pro	Tyr	Leu	Ala	Pro	Val	Ser	Pro	Trp	Phe	Phe	Thr	His	Phe	Gly	Pro	
		225					230					235				
GAA	GTT	TCA	TAT	TCC	AAG	AAC	TGG	GTC	TTC	CCA	GGT	GGT	CCT	CTG	ATC	3949
Glu	Val	Ser	Tyr	Ser	Lys	Asn	Trp	Val	Phe	Pro	Gly	Gly	Pro	Leu	Ile	
		240				245					250					
TAT	AAC	CGG	TGG	CAA	CAG	GTC	TTG	CAG	CAG	GGC	TTC	CCC	ATG	GTT	GAG	3997
Tyr	Asn	Arg	Trp	Gln	Gln	Val	Leu	Gln	Gly	Phe	Pro	Met	Val	Glu		
		255			260				265					270		
ATT	GTT	ACC	TGG	AAT	GAC	TAC	GGC	GAG	TCT	CAC	TAC	GTC	GGT	CCT	CTG	4045
Ile	Val	Thr	Trp	Asn	Asp	Tyr	Gly	Glu	Ser	His	Tyr	Val	Gly	Pro	Leu	
				275				280					285			
AAG	TCT	AAG	CAT	TTC	GAT	GAT	GGC	AAC	TCC	AAA	TGG	GTC	AAT	GAT	ATG	4093
Lys	Ser	Lys	His	Phe	Asp	Asp	Gly	Asn	Ser	Lys	Trp	Val	Asn	Asp	Met	
			290					295					300			
CCC	CAT	GAT	GGA	TTC	TTG	GAT	CTT	TCA	AAG	CCG	TTT	ATT	GCT	GCA	TAT	4141
Pro	His	Asp	Gly	Phe	Leu	Asp	Leu	Ser	Lys	Pro	Phe	Ile	Ala	Ala	Tyr	
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AAG	AAC	AGG	GAT	ACT	GAT	ATA	TCT	AAG	TAT	GTT	CAA	AAT	GAG	CAG	CTT	4189
Lys	Asn	Arg	Asp	Thr	Asp	Ile	Ser	Lys	Tyr	Val	Gln	Asn	Glu	Gln	Leu	
		320			325						330					
GTT	TAC	TGG	TAC	CGC	CGC	AAC	TTG	AAG	GCA	TTG	GAC	TGC	GAC	GCC	ACC	4237
Val	Tyr	Trp	Tyr	Arg	Arg	Asn	Leu	Lys	Ala	Leu	Asp	Cys	Asp	Ala	Thr	
		335			340					345				350		
GAC	ACC	ACC	TCT	AAC	CGC	CCG	GCT	AAT	AAC	GGA	AGT	GGC	AAT	TAC	TTT	4285
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				355				360					365			
ATG	GGA	CGC	CCT	GAT	GGT	TGG	CAA	ACT	ATG	GAT	ACC	GTT	TAT	GTT		4333
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			435					440					445			
TGC	GGT	ATC	TAC	AAT	TTC	AAC	CCA	TAT	GTT	GGC	ACC	ATT	CCT	GGC	GGC	4573
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Leu	His	Val	Thr	Thr	Cys	Gln	Ala	Lys	Pro	Ser	Leu	Gly	Thr	Asn	Pro	
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Asn	Tyr	Gly	Tyr	Cys	Pro	Pro	Gly	Pro	Cys	Lys	Cys	Thr	Ala	Phe	Gly		
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CACAGTGGAG	CAGCAACATT	CCCCATCATT	GCTTTCCCCA	GGGGCCTCCC	AACGACTAAA												5522
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AAGAGGGTCC	CCATCCATCA	AACCCAGTTC	AATAATAGCC	GAGATGCATG	GTGGAGTCAA												5642
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GC CGCGCGTC	CAGGTTCAAC	TCTCTCCTCT	AGCGCCTGAT	GCGGTATTTT	CTCCTTAGCG												5822
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TGCTCCCGGC	ATCCGCTTAC	AGACAAGCTG	TGACCGTCTC	CGGGAGCTGC	ATGTGTCAGA												6002
GGTTTTCCACC	GTCATCACCG	AAACGCGCGA															6032

## PATENT CLAIMS

1. A method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of:
  - 5 a) isolating a DNA sequence encoding a mutanase from a filamentous fungus,
  - b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, or replacing the mutanase (pre)pro-sequence with a
  - 10 (pre)pro-sequence comprising a kex2 or kex2-like site of another fungal enzyme,
  - c) cloning the DNA sequence obtained in step b) into a suitable expression vector.
- 15 2. The method according to claim 1, wherein the mutanase is obtained from the genus *Trichoderma*, preferably a strain of the species *T. harzianum*, especially the strain *T. harzianum* CBS 243.71.
- 20 3. The method according to claim 2, in which the mutanase DNA sequence is isolated from or produced on the basis of a nucleic acid library of *Trichoderma harzianum* CBS 243.71.
4. The method according to any of claims 1 to 3, wherein the
- 25 mutanase (pre)pro-sequence is replaced by the Lipolase® (pre)pro-sequence or the TAKA-amylase (pre)pro-sequence.
5. An expression vector comprising a mutanase gene and a DNA sequence encoding a pro-peptide with a kex2 site or kex2-like
- 30 site between the DNA sequences encoding said pro-peptide and the mature region of the mutanase.
6. The expression vector according to claim 5, further comprising an operably linked promoter sequence and/or a prepro-
- 35 sequence.

7. The expression vector according to claims 5 and 6, wherein the prepro-sequence comprise the original mutanase signal sequence, or the Lipolase® signal-sequence, or the TAKA pro-sequence and the original mutanase pro-sequence with a kex2 or  
5 kx2-like site, or the Lipolase® pro-sequence, or the TAKA pro-sequence.
8. The expression vector according to claim 7, wherein the promoter is the TAKA promoter or TAKA:TPI promoter.
- 10 9. The expression vector according to any claims 5 to 8, being the vector pMT1796.
10. A filamentous host cell for production of recombinant  
15 mutanase derived from a filamentous fungus being from the genus *Trichoderma*, such as a strain of *T. harzianum*, or the genus *Aspergillus*, such as a strain of *A. oryzae* or *A. niger*, or a strain of the genus *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum*, *Fusarium sulphureum*, *Fusarium*  
20 *cerealis*.
11. The host cell according to claim 10 wherein the host cell is a protease deficient of protease minus strain.
- 25 12. The host cell according to claim 11, wherein the host cell is the protease deficient strain *Aspergillus oryzae* JaL125 having the alkaline protease gene named "alp" deleted.
13. A process for producing a recombinant mutanase in a host  
30 cell, comprising the steps:
- a) transforming an expression vector comprising a mutanase gene with a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase into a suitable filamentous fungus host cell,
- 35 b) cultivating the host cell in a suitable culture medium under conditions permitting expression and secretion of an active mutanase,

c) recovering and optionally purifying the secreted active recombinant mutanase from the culture medium.

14. The process according to claim 13 wherein the recombinant expression vector is prepared according to the method of claim 1 to 4.

15. The process according to claim 13 and 14, wherein the filamentous host is a host cell according to any of claims 7 to 9.

16. An isolated recombinant mutanase produced according to the process according to any of claims 13 to 15.

17. A substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any contaminants.

18. A composition comprising a recombinant mutanase according to claim 16 or a substantially pure wild-type mutanase according to claim 17 and further other ingredients conventionally used in food, feed and/or pet food products.

19. An oral care composition comprising a recombinant mutanase according to claim 16 or a substantially pure wild-type mutanase according to claim 17, further comprising an enzyme selected from the group of dextranases, oxidases, peroxidases, haloperoxidases, laccases, proteases, endoglucosidases, lipases, amylases, and mixtures thereof.

20. An oral care product comprising a recombinant mutanase according to claim 16 or a substantially purified mutanase according to claim 17 or an oral care composition according to claim 19 and further comprising ingredients conventionally used in oral care products.

21. The oral care product according to claim 20, being a dentifrice, such as a toothpaste, tooth powder or a mouth wash.

22. Use of the recombinant mutanase according to claim 16 or the substantially purified mutanase according to claim 17 or an oral care composition of claim 19 or oral care product according to claims 20 and 21 for preventing the formation of dental plaque or removing dental plaque.

23. The use of the recombinant mutanase according to claims 16 or the substantially purified mutanase according to claim 17 or a oral care composition of claim 19 or oral care product according to claims 18 and 20 in oral care products for humans and/or animals.

24. Use of the composition according to claim 18, in food, feed and/or pet food products.

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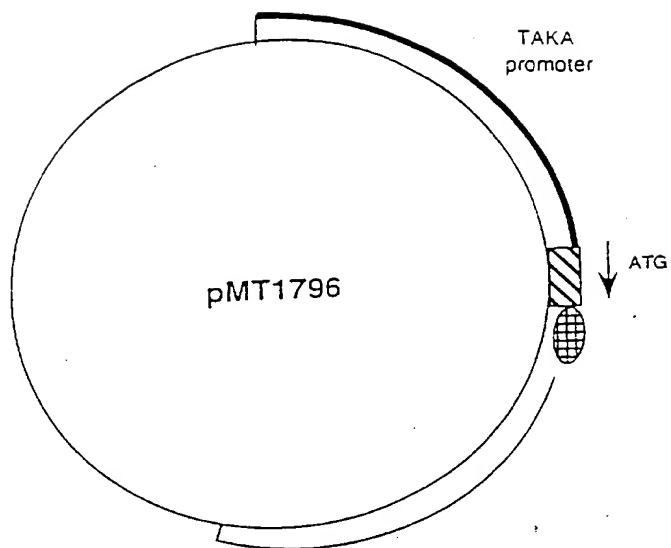
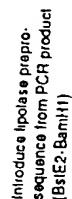


Fig. 1



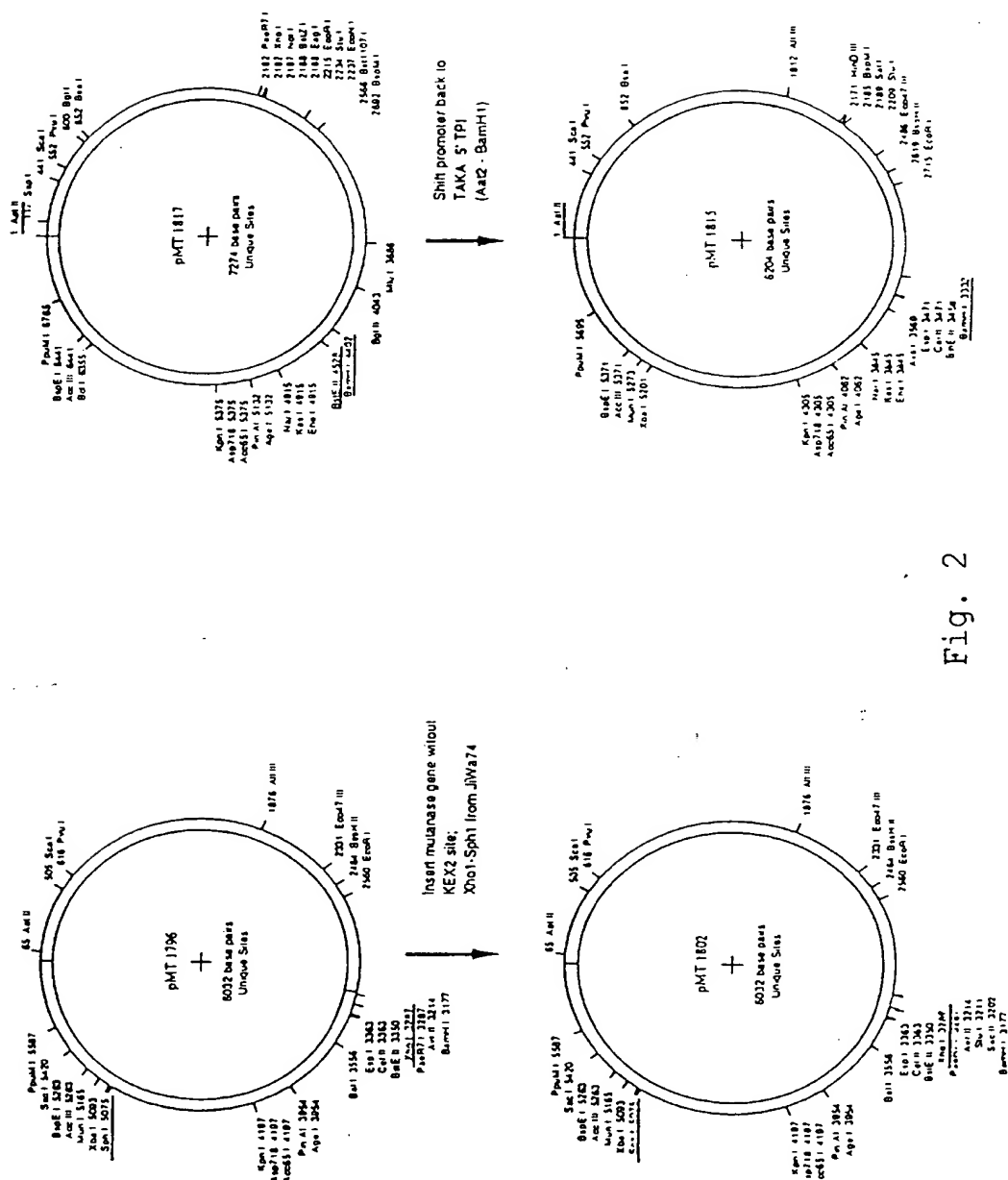


Fig. 2

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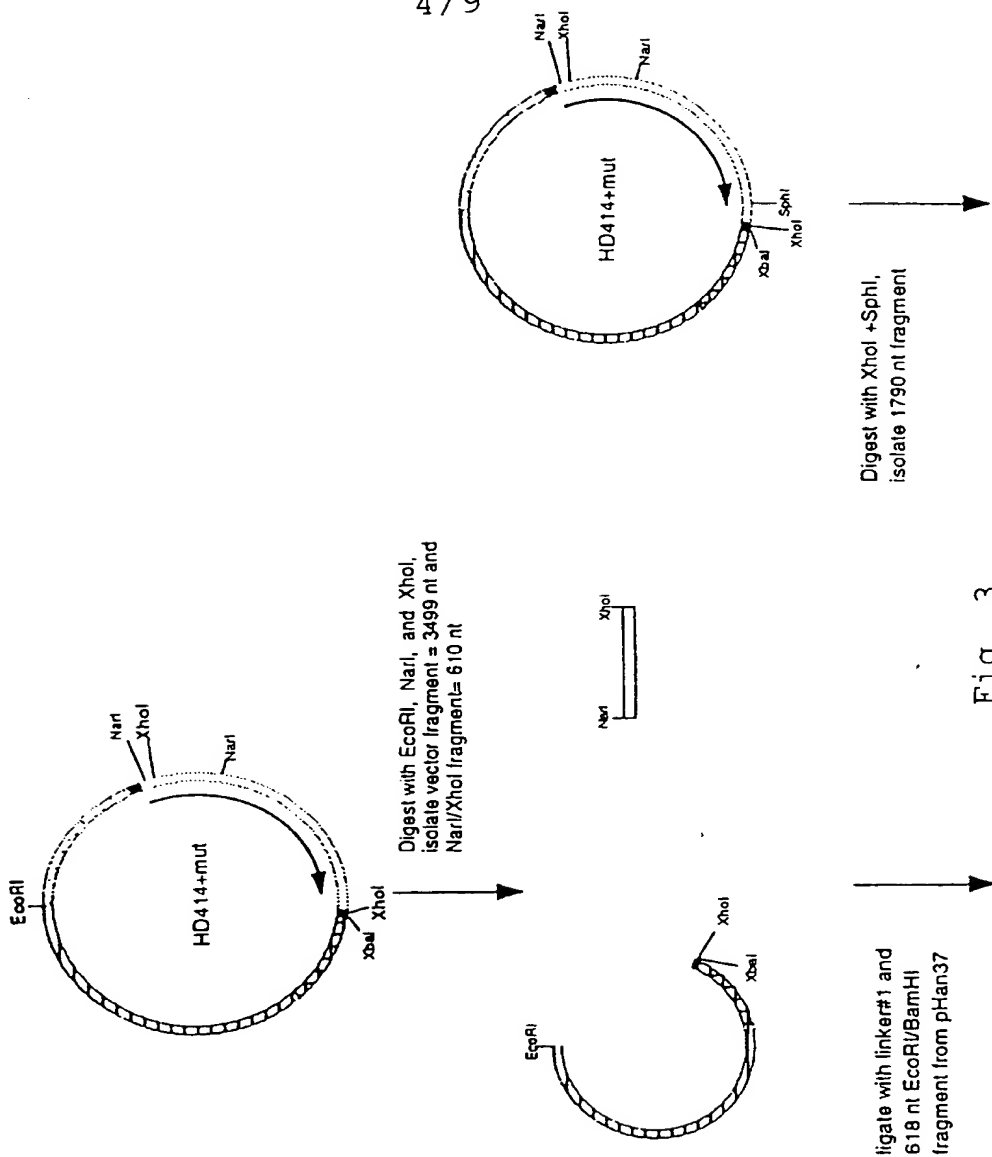


Fig. 3

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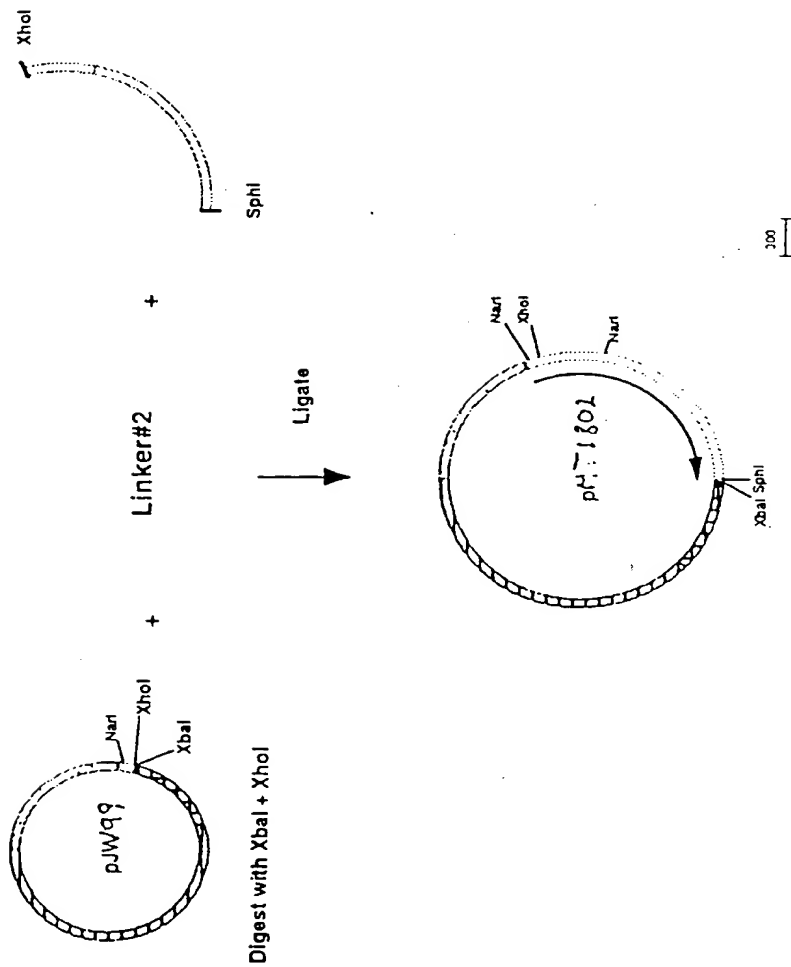


Fig. 3

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pH-profile of Mutanase in 50 mM BR at 40 °C

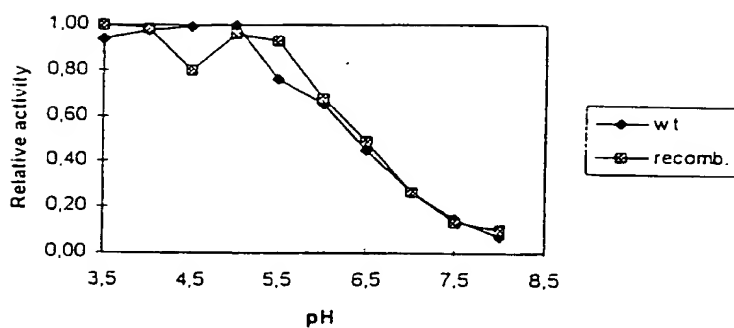


Fig. 4

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Temperature-profile of Mutanase in 0.1 M sodium phosphate, pH 7

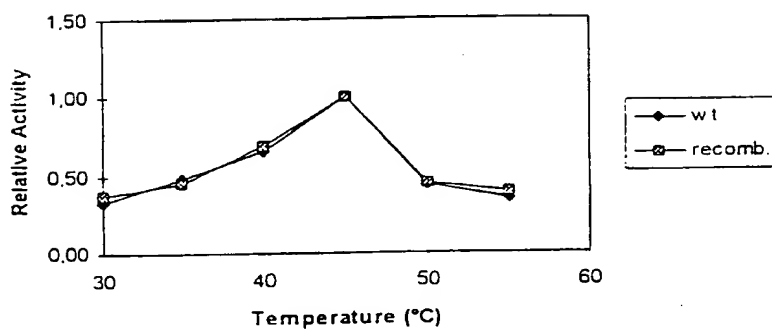


Fig. 5

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Temperature Stability of Mutanase in 0.1 M sodium phosphate, pH 7

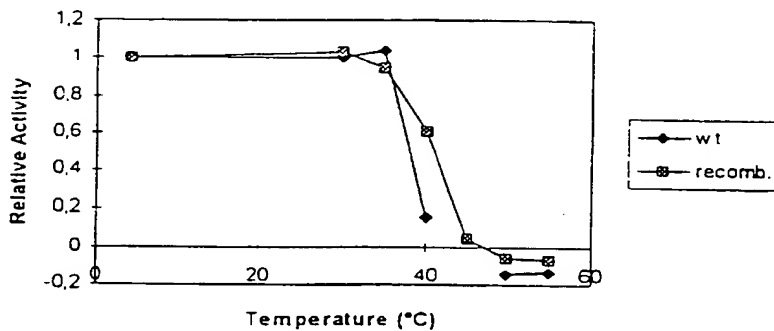


Fig. 6

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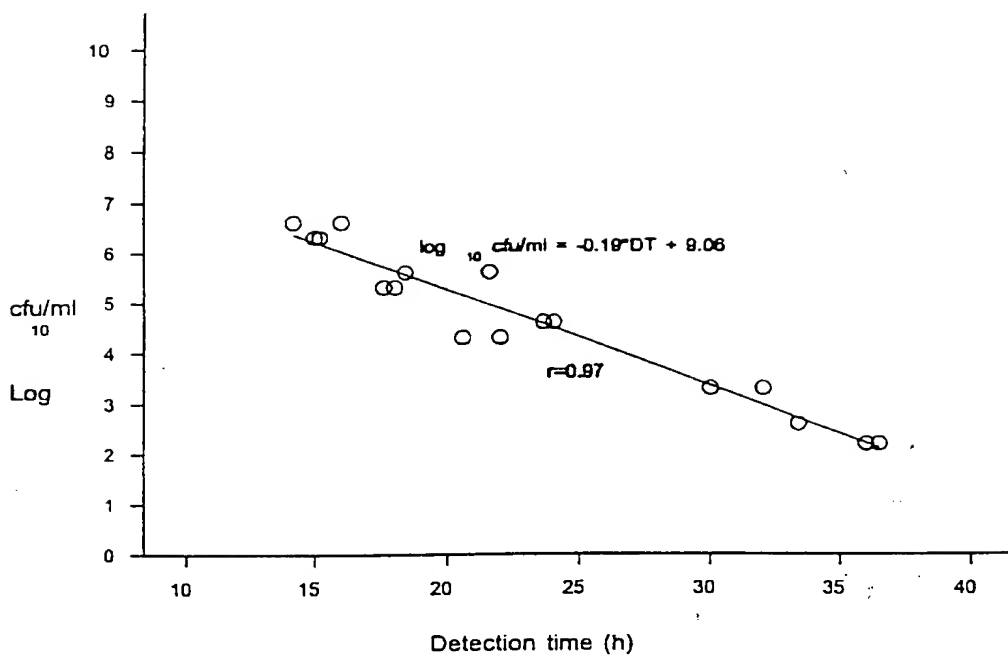


Fig. 7

1  
INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00283

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/24, C12N 15/56

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9602653 A1 (NOVO NORDISK BIOTECH, INC.), 1 February 1996 (01.02.96), see claims 5-6, 17 --	1-24
X	File WPI, Derwent accession no. 92-111673, NISSIN SHOKUHIN KAISHA LTD: "Alpha-1,3-glucanase gene - obt'd. by lighting DNA into vector, trans- forming it into oral cavity bacteria and secretion of enzyme protein"; & JP,A,4058889, 920225 --	1-24
X	US 4353891 A (BERNHARD GUGGENHEIM EET AL), 12 October 1982 (12.10.82), see column 4, line 6-63 --	17

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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Date of the actual completion of the international search

29 Sept. 1997

Date of mailing of the international search report

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2  
INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00283

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	US 5521093 A (YVES LEMOINE ET AL), 28 May 1996 (28.05.96)  --	1,5,7,13
A	EP 0327377 A2 (SUNTORY LIMITED), 9 August 1989 (09.08.89)  --	1,5,7,13
A	Dialog Information Services, file 155, MEDLINE, Dialog accession no. 07915749, Medline accession no. 94237475, Castelli LA et al: "High-Level secretion of correctly processed beta-lactamase from <i>Saccharomyces cerevisiae</i> using a high-copy- number secretion vector"; & Gene May 3 1994, 142 (1) p113-7  --	1,5,7,13
A	Chemical Abstracts, Volume 112, No 13, 26 March 1990 (26.03.90), (Columbus, Ohio, USA), page 238, THE ABSTRACT No 113593v, CN, 86102686 A,, (matsushiro, aizo) 21 October 1987 (21.10.87)  -- -----	1-24

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**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

01/09/97

International application No.

PCT/DK 97/00283

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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EP 0327377 A2	09/08/89	SE 0327377 T3 AT 123520 T CA 1335966 A DE 68922917 D,T ES 2072893 T JP 1199578 A US 5162220 A US 5234830 A	15/06/95 20/06/95 26/10/95 01/08/95 10/08/89 10/11/92 10/08/93
CN 86102686 A	21/10/87	NONE	

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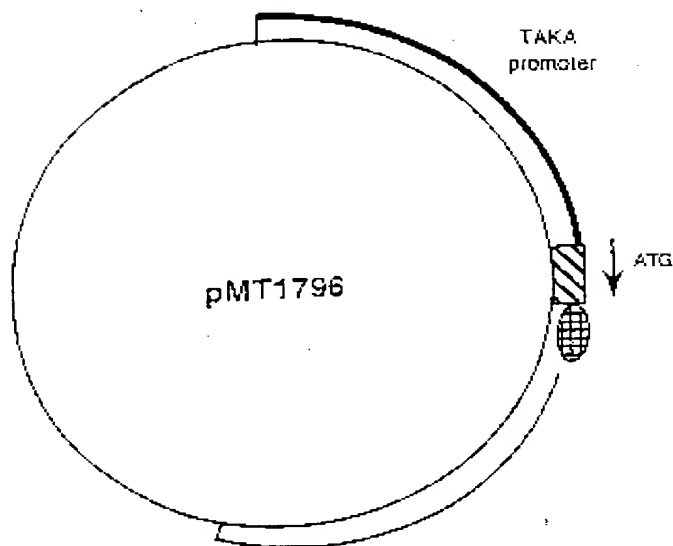


Fig. 1



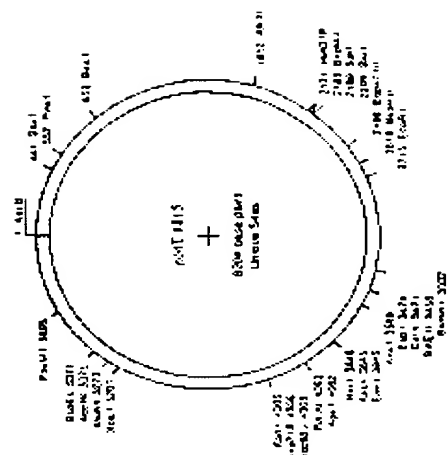
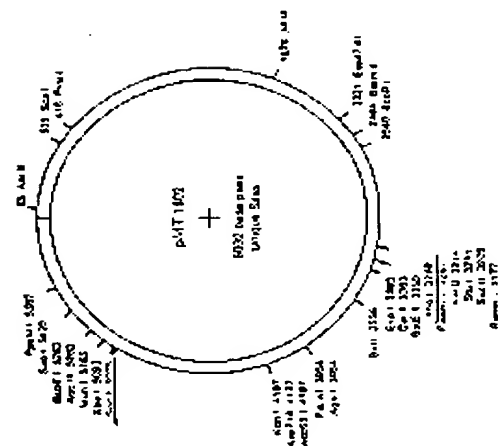


Fig. 2



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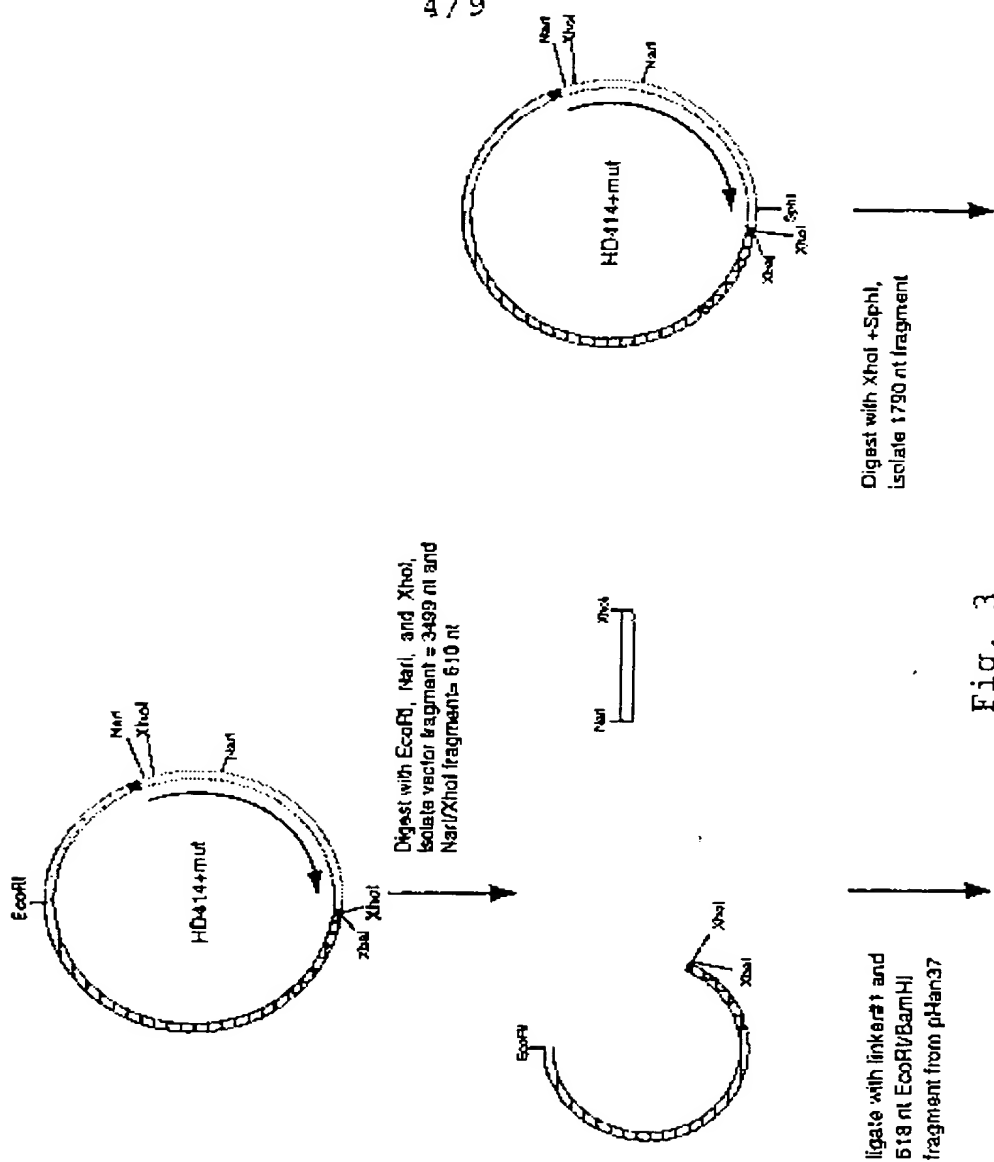


Fig. 3

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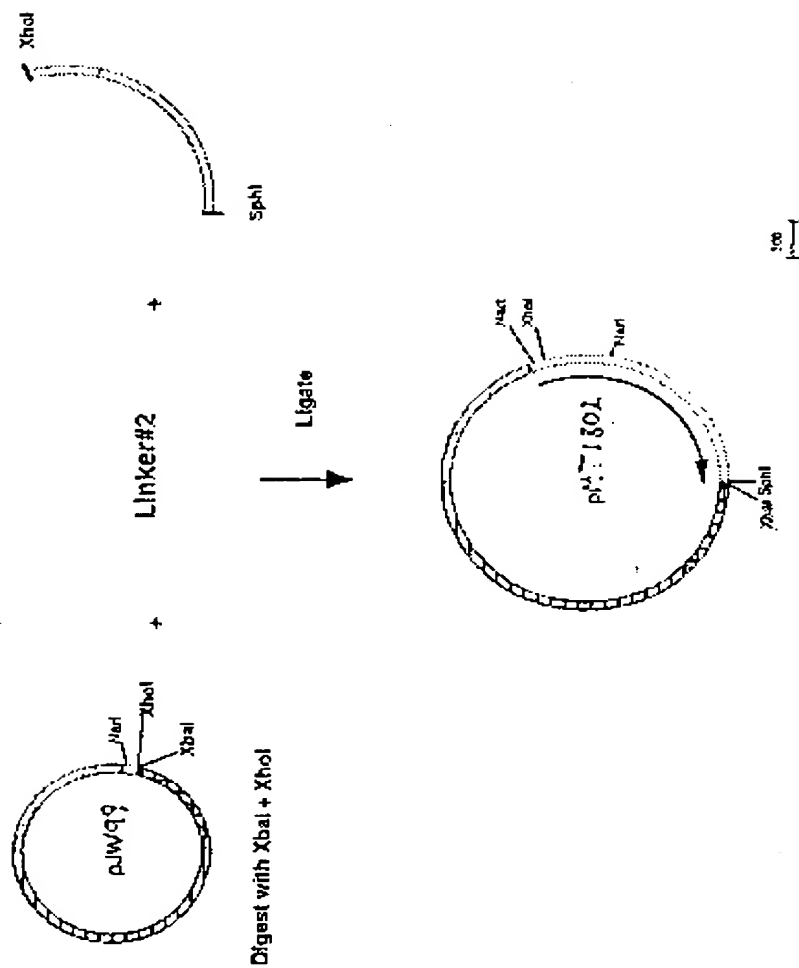


Fig. 3

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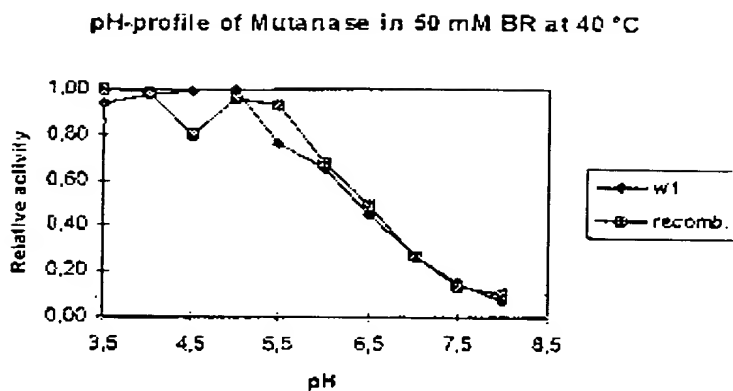


Fig. 4

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Temperature-profile of Mutanase in 0.1 M sodium  
phosphate, pH 7

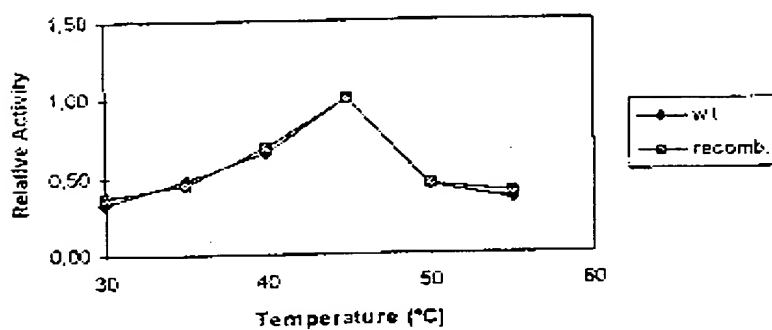


Fig. 5

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Temperature Stability of Mutanase in 0.1 M sodium phosphate, pH 7

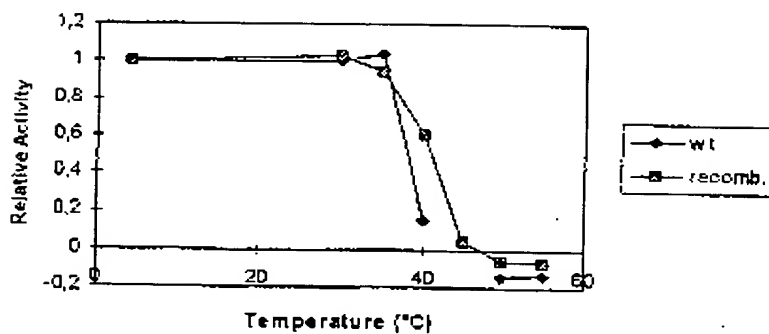


Fig. 6

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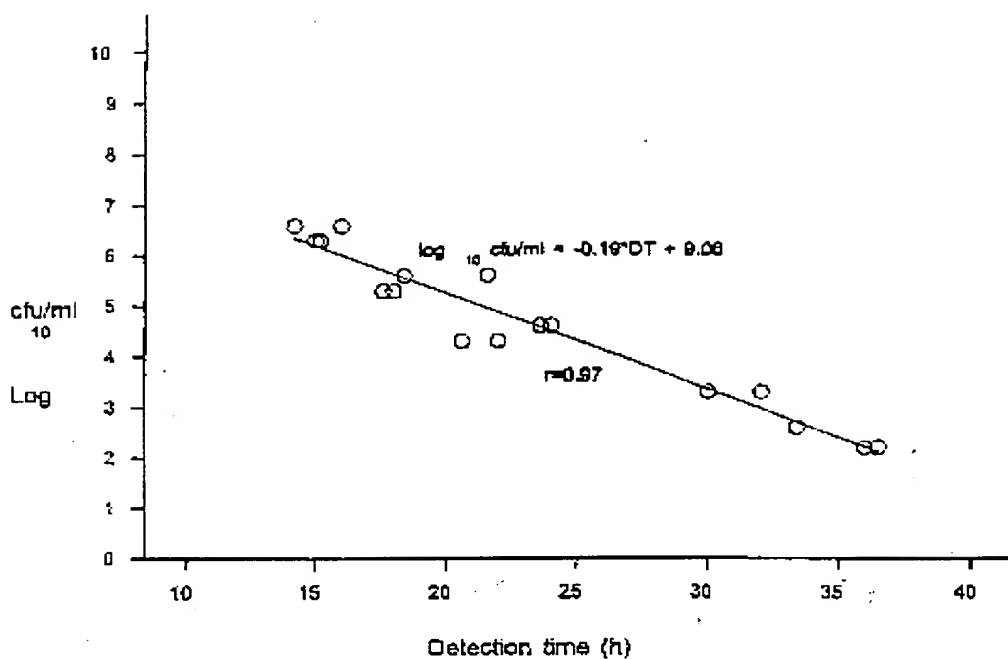


Fig. 7

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